GUIDELINES ON

STANDARD OPERATING PROCEDURES FOR

MICROBIOLOGY

World Health Organization
Regional Office for South-East Asia
New Delhi

Community Health Cell
Library and Information Centre
367, " Srinivasa Nilaya "
Jakkasandra 1st Main,
1st Block, Koramangala,
BANGALORE - 560 034.
Phone : 5531518 / 5525372

SEA/HLM/324
Distribution: General

Guidelines on Standard Operating Procedures for Microbiology

Sudarshan Kumari
Regional Advisor, Blood Safety & Clinical Technology
WHO, SEARO

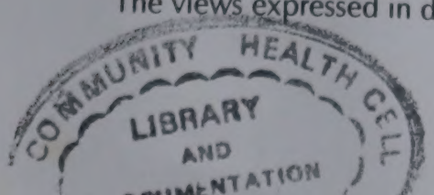
R.L. Ichhpujani
Consultant Microbiology and Head, Department of Zoonosis,
National Institute of Communicable Diseases, Delhi

World Health Organization
Regional Office for South-East Asia
New Delhi
May 2000

© World Health Organization 2000

This document is not a formal publication of the World Health Organization (WHO), and all rights are reserved by the Organization. The document may, however, be freely reviewed, abstracted reproduced or translated, in part or in whole, but not for sale or for use in conjunction with commercial purposes.

The views expressed in documents by named authors are solely the responsibility of those authors.



ST-100
03824

Acknowledgements

WHO gratefully acknowledges the valuable contributions of Dr Rajesh Bhatia, Consultant and Head, Department of Microbiology, National Institute of Communicable Diseases, Delhi; Dr C.S. Bhaskaran (former Director, Institute of Preventive Medicine, Hyderabad); Dr K.B. Sharma (former Regional Adviser, Health Laboratory Services, WHO Regional Office for South-East Asia) and Mr K.K. Khanna (former Joint Director, National Institute of Communicable Diseases, Delhi) to this publication.

Preface

Laboratory services have become an integral and inseparable component of modern medicine and public health. Laboratories play a decisive role in the diagnosis, treatment, prognosis and monitoring of both communicable and noncommunicable diseases. Intermediate and peripheral health facilities in developing countries are crucial to primary health care. Therefore, reliable, reproducible and rapid laboratory services, organized in a cost-effective manner, will go a long way in providing quality health services at the district hospitals and health centres.

Quality assurance in laboratory services, aimed at improving reliability, efficiency and facilitating inter-laboratory comparability in testing, is the backbone of quality health care delivery. The use of standard operating procedures in laboratory testing is one of the most crucial factor in achieving quality. This helps both in proper patient management and generates reliable disease surveillance data. This publication provides guidelines on standard operating procedures for diagnosing diseases of public health importance at intermediate and peripheral levels. Guidelines on early warning signals about epidemic-prone diseases based on laboratory data and on collecting and effectively transporting the appropriate clinical specimens to the referral/central laboratories for diseases for which diagnostic services are not as yet developed at the intermediate laboratories are also provided.

The publication contains guidelines on the use of conventional procedures which may be adapted as per local needs. The emphasis is on providing feasible, practical, easy-to-reproduce, specific, simple and cost-effective techniques for the diagnosis of communicable diseases. Necessary biosafety guidelines have been provided and situations where referrals to higher-level laboratories are indicated have also been identified.

It is hoped that this publication will be useful in achieving its objective of improving the quality of laboratory services at intermediate and peripheral levels.

Contents

		Page
Acknowledgements		i
Preface		iii
SECTION A: GENERAL LABORATORY PRACTICES		
1	**Organization and Functions of Laboratories**	1
	Peripheral laboratory services	1
	Intermediate laboratory services	3
2	**Collection and Transportation of Clinical Specimens**	5
	Criteria for rejection of specimens	5
	Collection of specimens	6
	Transportation of specimens	9
3	**Sterilization**	11
	Sterilization by heat	11
	Disinfection	13
	Biohazard waste management	15
4	**Staining Techniques**	17
	Methylene blue staining	17
	Gram staining	18
	Albert's staining	19
	India ink staining	20
	Ziehl Neelsen staining	20
	Iodine staining for ova and cysts	21
	Quality control of stains	22
5	**Bacteriological Media**	23
	Types of media	23
	Preparation of media and checking of pH	24
	Nutrient broth	24
	Nutrient agar	25
	Glucose broth	25
	Blood agar	25
	Chocolate agar	25
	XLD agar	25
	Buffered glycerol saline	26
	Loeffler serum medium	26
	Blood tellurite agar	27
	Salt broth (10%)	27
	Peptone water	28
	Alkaline peptone water	28
	MacConkey agar	28

		Page
	Bile salt agar	28
	Selenite F broth	30
	Media for carbohydrate fermentation	30
	Lowenstein Jensen medium	31
	Cary and Blair transport medium	32
	Stuart transport medium	32
	Venkataraman-Ramakrishnan (V.R.) holding medium	33
	MacConkey broth for bacteriological examination of water	33
	Sabouraud Dextrose Agar (SDA)	34
	Performance of plated media	36
6	**Cultivation of Bacteria on Laboratory Media**	39
	Instrument for seeding media	39
	Aseptic techniques	41
7	**Antimicrobial Susceptibility Testing**	43
	Modified Kirby-Bauer method	44
	Quality assurance in susceptibility test	47
	Biosafety	48
	Referral	48
	When intermediate laboratories need not undertake suscpetibility tests?	48
	Salient features of quality assurance in antibiotic susceptibility testing	48
8	**Safety in Laboratories**	53
	Laboratory biosafety levels	53
	Preventive measures against laboratory infections	54
	Pipetting	54
	Hypodermic syringes and needles	54
	Opening containers	55
	Laboratory access	56
	Clothing	56
	Accidents in laboratory	56
	Accidents and spills	57
	Management of laboratory accidents	57
	General laboratory directions for safety	58
9	**Quality Assurance**	59
	What is the objective of QA?	59
	Factors affecting the quality	59
	Maintenance of equipment	61
	Performance tests on culture media	62
	Quality control for commonly-used tests	63
	Quality control of immunological tests	63
	QA of antibiotic susceptibility testing	63
	In service training of staff	65
	Participation in external quality assessment	65
	SECTION B: STANDARD PROCEDURES FOR SPECIFIC DISEASES	
10	**Cholera**	67
	Causative organism	67
	Specimens	67
	Collection, storage and transportation of specimens	67

		Page
	Materials	68
	Procedure	69
	Quality assurance	72
	Antimicrobial susceptibility testing	72
	Reporting of results	72
	Referral	72
	Biosafety	72
11	**Diphtheria**	73
	Collection and transportation of specimens	73
	Storage	74
	Processing of swabs	74
	Staining of smears	74
	Reporting of results	76
	Quality assurance	76
	Other significant organisms isolated from throat swabs	76
	Important points about diagnosis of diphtheria	76
	Susceptibility to antimicrobial agents	77
	Biosafety	77
	Referral	77
12	**Enteric Fever**	79
	Collection of specimen	79
	Isolation of the organism	79
	Antimicrobial susceptibility testing	81
	Serodiagnosis	81
	Quality assurance	83
	Diagnosis of chronic typhoid carriers	84
	Biosafety	84
	Referral	84
13	**Pyogenic Meningitis**	87
	Causative organisms	87
	Collection, storage and transportation of specimens	88
	Abnormalities associated with pyogenic meningitis	89
	Immunological tests	91
	Antimicrobial susceptibility testing	92
	Quality assurance	93
	Biosafety	93
	Referral	93
14	**Dysentery**	95
	Collection of faeces	95
	Macroscopic examination	96
	Microscopic examination	96
	Isolation of organism	96
	Cautions	97
	Biosafety	97
	Referral	97
	Antimicrobial sensitivity testing	97
	Reporting of results	97
	Quality assurance	98

		Page
15	**Gonorrhoea**	99
	Causative organism	99
	Specimen collection	99
	Staining the smear and examination	100
	Specimen transportation for culture	101
	Culture	101
	Presumptive identification	102
	Biosafety	103
	Quality assurance	103
	Reporting of results	103
	Antimicrobial susceptibility testing	103
	Referral	104
16	**Syphilis**	105
	Direct demonstration	105
	Reporting of results	107
	Susceptibility testing	107
	Serological evidence	107
	Biological false positives in VDRL	109
	Quality assurance in VDRL testing	110
	Referral	110
	Biosafety	110
17	**Tuberculosis**	113
	Morphology of M.tuberculosis	113
	Microscopy of sputum	113
	Collection of sputum sample	113
	Storage and transportation of specimen	114
	Preparation of smear and Ziehl Neelsen staining (AFB staining)	114
	Culture for M. tuberculosis	115
	Biosafety	116
	Quality assurance	117
	Susceptibility to antituberculosis drugs	117
	Reporting of results	117
	Referral	117
18	**Malaria**	119
	Preparation of blood smear	119
	Recognition of the malaria parasite	121
	Common defects in making blood films	123
	Reporting of results	123
	Referral	123
	Quality assurance	123
	Biosafety	124
19	**Urinary Tract Infection**	127
	Causative organisms	127
	Specimen	127
	Specimen transport	129
	Culture	129
	Quality assurance	130

Page

Susceptibility to antimicrobial agents 130
Reporting of results 130
Referral 131
Biosafety 131

20 **Parasitological Examination of Faeces** 133
Collection of faecal sample 133
Transportation of samples 134
Macroscopic examination 134
Microscopic examination (temporary wet mounts) 134
Concentration techniques 135
Formal ether sedimentation technique 136
Biosafety 138
Disposal of morbid material 138
Quality assurance 138
Reporting of results 138
Referral 138

21 **Mycological Techniques** 141
Collection and processing of samples 141
Presumptive identification based on direct microscopy 143
Biosafety 145
Quality assurance 145
Reporting 146
Referral 146

22 **Bacteriological Examination of Water** 147
Microbiological examination of water 147
Frequency of examination 151
Standards 151
Reporting 152
Referral 152
Quality assurance 152

SECTION C: COLLECTION AND TRANSPORTATION OF CLINICAL MATERIAL TO REFERRAL LABORATORIES

23 **Bacterial Food Poisoning** 153
Causative agents 153
Collection of specimen 153
Transportation of specimens 154
Interpretation 155
Referral 155
Quality assurance 155
Biosafety 156

24 **Acquired Immunodeficiency Syndrome** 157
Collection of sample of blood 158
Quality assurance 159
Referral 159
Biosafety 160
Reporting of results 160

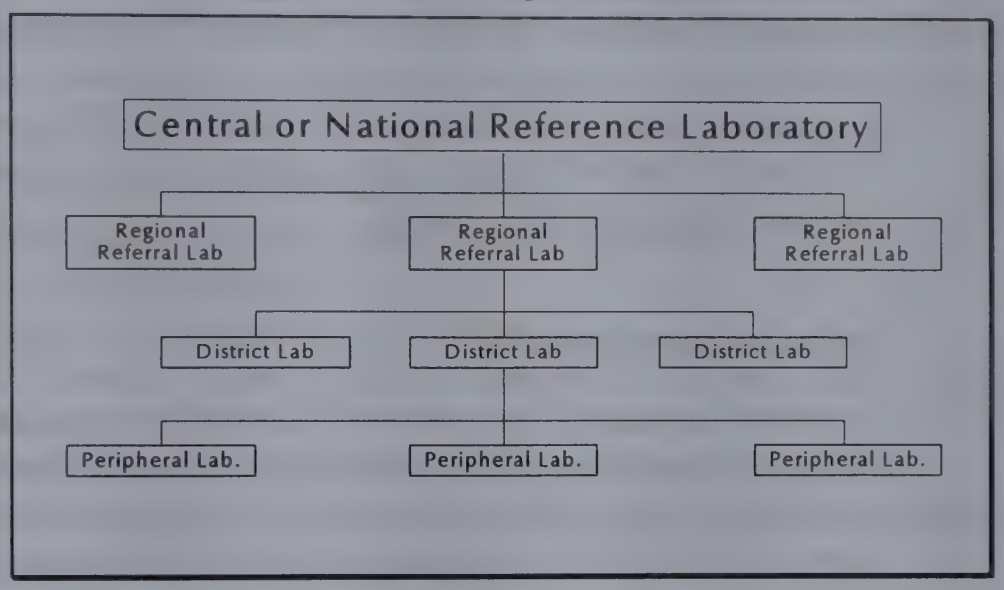
		Page
25	**Viral Hepatitis**	163
	Collection and transportation of specimens	163
	Reporting of results	165
	Quality assurance	165
	Biosafety	166
	Referral	166
26	**Poliomyelitis**	167
	Collection of stool specimen from a case with acute flaccid paralysis (poliomyelitis)	167
	Reporting of results	168
	Referral	169
	Quality assurance	169
27	**Dengue Fever and Dengue Haemorrhagic Fever**	173
	Laboratory diagnosis	173
	Recognizing cases of DF/DHF/DSS	175
	Reporting of results	176
	Quality assurance	176
	Biosafety	176
	Referral	176
Index		177

Section A
General Laboratory Practices

1. Organization and Functions of Laboratories

The organization of laboratories in any country is usually a three or four tier system with various possible functional linkages between them. One possible way of networking of laboratories is shown in Fig 1.

Figure 1: *Networking of Laboratories*



Peripheral laboratory services

Peripheral laboratories are located at the point of first contact of patients with the health care services. In most developing countries these are available only at primary health centre or community health centre (upgraded primary health centre) level. These laboratories provide technical support for preventive, curative and promotive services for the individual as well as the community.

Staff

The staff in peripheral laboratories should include one technician and one laboratory assistant/attendant.

Space

The space available in peripheral laboratories should include at least one laboratory-cum-office/record room (approx. 5 meters x 3 meters) and one store-room which can be used for other services also (approx. 5 meters x 3 meters).

Other facilities

Other necessary facilities include

- supply of safe water
- reliable source of energy (battery, electricity, solar or kerosene)
- sterilization/disinfection facilities
- waste disposal facilities

There must also be transport and communication facilities between the peripheral and intermediate laboratories for referral of samples and patients, procurement of supplies and personal discussion.

Equipment and supplies

Necessary equipment and supplies include good microscopes, centrifuges, autoclaves, refrigerators, balances, pH meters, incubators, water bath, transport media, glassware, sterile swabs, reagents for staining (eg. Gram, Albert, Ziehl Neelsen, Romanowsky), reagents for chemical examination of urine, kits and reagents for rapid diagnostic tests, sterilized syringes and needles, micropipettes and tips as well as sterile collection bottles for blood/serum and water analysis.

Tests to be performed

Peripheral laboratories are expected to undertake tests of public health as well as clinical relevance. Among the tests of public health relevance, diseases of greater epidemiological importance should be accorded priority. Testing of environment samples (especially water) also falls into the priorities of public health relevance. Certain rapid serological tests may be of use in studying epidemiological patterns of important diseases and the same can also be performed at peripheral laboratories.

The tests to be performed by peripheral laboratories are subject to the availability of resources, manpower, technology and prevalence of various diseases in the area catered to by the laboratory. A suggested list is provided in Table 1.

Table 1: ***Suggested tests to be performed at peripheral laboratories***

Procedure/Specimen	For detection/diagnosis of
Urine examination	Pus cells, RBCs Albumin Sugar
Stool examination	Ova and cysts
Stained smears	
Throat specimen	Diphtheria
Sputum	Tuberculosis
CSF (pyogenic and tubercular)	Meningitis
Peripheral blood smear	Malaria, filariasis
Rapid diagnostic tests	HIV Hepatitis B surface Ag Syphilis Meningococcal disease

Intermediate laboratory services

In most developing countries, intermediate laboratories are located at district or the regional headquarters and may act as clinical as well as public health laboratories. The following functions are expected to be performed by these laboratories:

1. Laboratory support to clinical diagnosis/public health
   - Quality assurance
   - Logistic and technical support
   - Training of staff for peripheral laboratories

2. Supervision and monitoring of peripheral laboratories

Intermediate laboratories help in the diagnosis and treatment of the individual patient and are also used as public health laboratories for epidemiological surveillance and control of diseases in the community. These laboratories also serve as links between peripheral laboratories and the state/central laboratory for the following:

- ➢ Collection, storage and analysis of data.
- ➢ Distribution of reagents, media, laboratory manuals.
- ➢ Purchase of equipment.
- ➢ Supervision of peripheral laboratories.
- ➢ To conduct external quality assessment scheme (EQAS) for peripheral laboratories.
- ➢ To take part in EQAS organized by the state/central laboratories.
- ➢ To send samples to higher/reference laboratories for characterization of isolate/confirmation of diagnosis.

Staff

Qualified pathologist/ microbiologist (Doctor of Medicine/diploma in clinical pathology)	1
Technicians –	
DMLT (diploma in medical laboratory technology) with experience	2
Laboratory Assistants (DMLT)	1
Laboratory attendants	2
Cleaner	1
Clerk-cum-storekeeper	1

Since it may not be possible to have a full-time epidemiologist, at least part time help of an epidemiologist should be available.

Space

Microbiology/Serology laboratory (approx.8 metersx5 meters)	1
Sterilization, disinfection and media preparation laboratory (approx. 6 metersx4 meters)	1
Store-room (approx.3 metersx5 meters)	1
Office (approx. 3 metersx5 meters)	1

Equipment

Binocular microscope	2	Colorimeter	1
Dark-field microscope	1	Refrigerator	1
Inoculating chamber	2	Balances	2
Centrifuge	2	pH meter	1
Autoclave	2	Inspissator	1
Incubator	2	Distil water apparatus	1
Hot air oven	1	Micropipettes	as per workload
Water bath	2	Tips for pipettes	as per workload
VDRL shaker	1		

This manual describes most of the tests that have been suggested to be performed at intermediate-level laboratories.

Further reading

1. Kumari S, Bhatia Rajesh, Heuck CC. Quality Assurance in Bacteriology and Immunology. WHO Regional Publication, South East Asia Series No 28, 1998.
2. Kumari S, Sharma KB et al. Health Laboratory Services in support of Primary Health Care in South-East Asia, WHO Regional Publication, South East Asia Series No 24, 2nd Ed, 1999, New Delhi.

2. Collection and Transportation of Clinical Specimens

The laboratory diagnosis of an infectious disease begins with the collection of a clinical specimen for examination or processing in the laboratory (the *right* one, collected at the *right* time, transported in the *right* way to the *right* laboratory). Proper collection of an appropriate clinical specimen is the first step in obtaining an accurate laboratory diagnosis of an infectious disease. Guidelines for the collection and transportation of specimens should be made available to clinicians in a lucidly written format. The guidelines must emphasize two important aspects:

- ➢ Collection of the specimen before the administration of antimicrobial agents.
- ➢ Prevention of contamination of the specimen with externally present organisms or normal flora of the body.

General rules for collection and transportation of specimens are summarized in Table 1.

Table1: ***Collection and transportation of specimens***

- Apply strict aseptic techniques throughout the procedure.
- Wash hands before and after the collection.
- Collect the specimen at the appropriate phase of disease.
- Make certain that the specimen is representative of the infectious process (e.g. sputum is *the* specimen for pneumonia and not saliva) and is adequate in quantity for the desired tests to be performed.
- Collect or place the specimen aseptically in a sterile and/or appropriate container.
- Ensure that the outside of the specimen container is clean and uncontaminated.
- Close the container tightly so that its contents do not leak during transportation.
- Label and date the container appropriately and complete the requisition form.
- Arrange for immediate transportation of the specimen to the laboratory.

Criteria for rejection of specimens

Criteria should be developed by a laboratory on the basis of which the processing of a specimen may not be done by the laboratory. The following are some examples:

- ➢ Missing or inadequate identification.
- ➢ Insufficient quantity.
- ➢ Specimen collected in an inappropriate container.

- Contamination suspected.
- Inappropriate transport or storage.
- Unknown time delay.
- Haemolysed blood sample.

Collection of specimens

The clinical state of the patient will not necessarily be reflected by the result of laboratory investigation despite correct laboratory performance unless the specimen is in optimal condition required for the analysis. Some of the important specimens and their proper collection and transportation methods are described here so as to ensure quality.

Blood

Whole blood is required for bacteriological examination. Serum separated from blood is used for serological techniques. Skin antisepsis is extremely important at the time of collection of the sample. Tincture of iodine (1-2%), povidone iodine (10%) and chlorhexidine (0.5% in 70% alcohol) are ideal agents. However, some individuals may be hypersensitive to iodine present in some of these. While collecting blood for culture,the following points must be remembered:

- Collect blood during the early stages of disease since the number of bacteria in blood is higher in the acute and early stages of disease.
- Collect blood during paroxysm of fever since the number of bacteria is higher at high temperatures in patients with fever.
- In the absence of antibiotic administration, 99% culture positivity can be seen with three blood cultures.
- Small children usually have higher number of bacteria in their blood as compared to adults and hence less quantity of blood needs to be collected from them (Table 2).

Table 2: ***Volume of blood to be collected at different ages***

Age	Volume in 2 bottles
< 2 years	2 ml
2-5 years	8 ml
6-10 years	12 ml
>10 years	20 ml

Cerebrospinal fluid (CSF)

Examination of CSF is an essential step in the diagnosis of any patient with evidence of meningeal irritation or affected cerebrum. Almost 3-10 ml of CSF is collected and part of it is used for biochemical, immunological and microscopic examination and remaining for bacteriological or fungal examination. The following important precautions need to be taken for CSF collection and transportation:

- Collect CSF before antimicrobial therapy is started.
- Collect CSF in a screw – capped sterile container and not in an injection vial with cotton plug.
- Do not delay transport and laboratory investigations.
- Transport in a transport medium if delay in processing is unavoidable.
- CSF is a precious specimen, handle it carefully and economically. It may not be possible to get a repeat specimen.
- Perform physical inspection immediately after collection and indicate findings on laboratory requisition form.
- Store at 37°C, if delay in processing is inevitable.

The characteristics of the appearance of CSF are outlined in Table 3.

Table 3: ***Appearance and interpretations of CSF***

Clear and colourless	Normal
Clear with Tyndall effect *(sparkling appearance against incident light)*	High protein content
Clear yellowish	Old haemolysis
Clear red	Fresh haemolysis
Turbid blood-stained	Haemorrhage
Turbid white	High cell or protein content
Turbid clot *(after overnight storage)*	Fibrin clots

Sputum

Sputum is processed in the laboratory for aetiological investigation of bacterial and fungal infections of the lower respiratory tract. It is of utmost importance in the diagnosis of pulmonary tuberculosis.

- Select a good wide-mouthed sputum container, which is preferably disposable, made of clear thin plastic, unbreakable and leak proof material.
- Give the patient a sputum container with the laboratory serial number written on it. Show the patient how to open and close the container and explain the importance of not rubbing off the number written on the side of the container.
- Instruct the patient to inhale deeply 2-3 times, cough up deeply from the chest and spit in the sputum container by bringing it closer to the mouth.
- Make sure the sputum sample is of good quality. A good sputum sample is thick, purulent and sufficient in amount (2-3 ml).

Give the patient an additional container with laboratory serial number written on it for an early morning specimen. Explain to the patient to rinse his/her mouth with plain water before bringing up the sputum.

Urine

Under normal circumstances urine is sterile. The lower part of the urethra and the genitalia are normally colonised by bacteria, many of which may also cause urinary tract infection. Since urine is a good growth medium for all sorts of bacteria, proper and aseptic collection assumes greater importance for this specimen.

For microbiological examination urine must be collected as a "clean catch-mid-stream" specimen.

Urine specimens should be transported to the laboratory within one hour for bacteriological examination, because of the continuous growth of bacteria *in vitro* thus altering the actual concentration of organisms.

Stool

Faecal specimens for the aetiological diagnosis of acute infectious diarrhoeas should be collected in the early stage of illness and prior to treatment with antimicrobials. A stool specimen rather than a rectal swab is preferred.

- The faeces specimen should not be contaminated with urine.
- Do not collect the specimen from bed pan.
- Collect the specimen during the early phase of the disease and as far as possible before the administration of antimicrobial agents.
- 1 to 2 gm quantity is sufficient.
- If possible, submit more than one specimen on different days.
- The fresh stool specimen must be received within 1-2 hours of passage.
- Store at 2-8°C.
- Modified Cary and Blair medium (see chapter 5) is recommended as a good transport medium. It is a very stable medium and can be stored for use in screw – capped containers. It is a semi-solid transport medium. At least two swabs should be inoculated. Most pathogens will survive for up to 48 hours at room temperature. Specimens are unacceptable if the medium is held for more than one week or if there is detectable drying of the specimen.

  Alternative transport media are Venkataraman-Ramakrishnan medium (V-R fluid) or alkaline peptone water. VR fluid should be prepared in 30 ml (1 oz) screw capped bottles (MacCartney bottles). It preserves vibrios for more than six weeks and has also proved to be a very convenient medium for transportation as it can be kept at room temperature after collection of the specimen.

Throat swab

- Depress the tongue with a tongue blade.
- Swab the inflammed area of the throat, pharynx or tonsils with a sterile swab taking care to collect the pus or piece of membrane.
- Transport in sterile transport tube.

Bone marrow

Bone marrow is collected by a doctor who is well trained in this procedure

- Decontaminate the skin overlying the site from where specimen is to be collected with 70% alcohol followed by 2% tincture of iodine.
- Aspirate 1 ml or more of bone marrow by sterile percutaneous aspiration.
- Collect in a sterile screw-cap tube.
- Send to laboratory immediately.

Rectal swab

- Insert swab at least 2.5 cm beyond the anal sphincter so that it enters the rectum.
- Rotate it once before withdrawing.
- Transport in Cary and Blair or other transport medium.

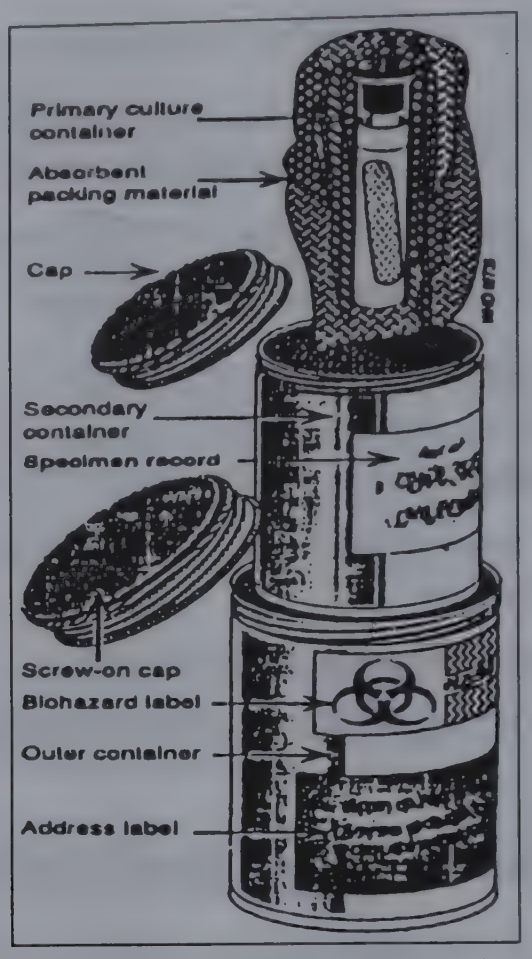
Transportation of specimens

Specimens to be sent to other laboratories require special attention for safe packing of the material. Guidelines are usually issued by national authorities and the same should be strictly followed. For hand-carried transportation over a short distance, the specimen should be placed upright in appropriate racks. For long distance transportation, it should be placed in three containers, i.e:

- **A primary container** which has the specimen and is leakproof with a screw-cap.
- **A secondary container** which is durable, waterproof and made of metal or plastic with a screw-cap. It should have enough absorptive material to absorb the contents of the primary container should the latter break or leak. On its outside, the details of the specimen should be pasted.
- **A tertiary container** is usually made of wood or cardbox. It should be capable of withstanding the shocks and trauma of transportation. Dry ice can be kept between this and the secondary container along with sufficient absorbents and provision for the escape of carbondioxide to prevent a pressure build-up inside (Fig 1).

In general, most specimens should be processed in the laboratory within 1 to 2 hours after collection. In practice, a 2-to 4-hour time limit is probably more practical during a normal working day. The laboratory must be organized to permit processing of the specimens as soon as they arrive, and the collection of most specimens should be limited to the working hours of the laboratory. However, some arrangements must be made to allow for the initial handling of the few specimens that have to be collected outside of the laboratory's working hours.

Figure 1: Transportation container



A continuous effort must be made in order to ensure proper collection and transportation of clinical specimens. Full cooperation of nursing staff and others concerned with specimen collection is required and can be achieved once they are made aware of the principles involved and the significance of what they are being asked to do.

Further reading

1. Lennette HE, Balows A, Hauser WJ et al. Collection, Handling and Processing of Specimen. In Manual of Clinical Microbiology, 4th Ed, ASM, Washington, DC, 73-98, 1985.

2. El-Nageh, Heuck CC, Appel W, Vandepitte et al. Basics of quality assurance in peripheral laboratories. WHO EMRO Series No 2, Alexandria, 1992.

3. Sterilization

Sterilization is defined as the destruction or removal (by filtration) of all microorganisms and their spores, whereas **disinfection** is the destruction of many microorganisms but not usually the bacterial spores. Sterilization is usually achieved with the help of heat whereas chemical agents are employed to effect disinfection.

Sterilization and disinfection are part of the daily routine of microbiological laboratories and constitute a vital activity which ensures that cultures, containers, media and equipment are treated in such a way that only the inoculated organisms will grow while all others will be eliminated.

Sterilization by heat

This can be achieved by autoclaving, by exposing articles to dry heat in hot air ovens or boiling.

Autoclaving

Autoclaves can sterilize anything that can withstand a temperature of 121°C for 30 minutes. A pressure cooker used in homes for cooking purposes can also be used as a makeshift autoclave.

The containers having clinical material are subjected to heat treatment in the autoclave after which these are emptied and washed and put back into service.

Only autoclaves designed for laboratory work and capable of dealing with a mixed load should be used. Porous load and bottle fluid sterilizers are rarely satisfactory for laboratory work. There are two varieties of laboratory autoclaves:

- Pressure cooker type.
- Gravity displacement models with automatic air and condensate discharge.

Pressure-cooker type laboratory autoclaves

The most common type is a device for boiling water under pressure. It has a vertical metal chamber with a strong metal lid which can be fastened down and sealed with a rubber gasket. An air and steam discharge tap, pressure gauge and a safety valve are fitted in the lid. Water in the bottom of the autoclave is heated by external gas burners, an electric immersion heater or a steam coil.

Operating instructions

- Ensure that there is sufficient water inside the chamber.
- Load the autoclave and fasten the lid keeping the discharge tap open.
- Adjust the safety valve to the required temperature and turn the heat on.
- Allow the mixture of air and steam to pass out freely till all air has been discharged.
- Close the air discharge tap and let the steam pressure rise within the chamber till it attains a temperature of 121°C (1.5 kg/cm^2).
- Hold on the pressure for 15 minutes.
- Turn off the heat and let the autoclave cool.
- Slowly open the air and steam discharge taps after the pressure gauge has reached zero.
- Allow the material to cool before these are handled (usually agar bottles take hours before these become safe to handle).

Autoclave with air discharge by gravity displacement

These are usually rectangular in shape and arranged horizontally. These autoclaves have a jacket around the chamber.

Operating instructions

- Bring the jacket of the autoclave to operating temperature.
- Load the chamber, close the door and open the steam valve so that steam can freely enter the top of the chamber. Air and condensate shall automatically flow out through the drain at the bottom.
- When the drain thermometer reaches the required temperature, allow further period for the load to reach that temperature (this has to be determined initially and periodically for each autoclave).
- Continue the autoclave cycle for the holding period.
- Close the steam valve and let the autoclave cool till a temperature of 80°C is reached.
- Gradually and softly open the autoclave enabling the steam to escape and allow the load to cool further.

Hot air oven

A hot air oven is electrically operated and should be equipped with a fan to ensure uniform temperature inside. The required temperature for sterilization is generally 160°C for one hour.

Operating instructions

- Arrange the material to be sterilized loosely and evenly on the racks of the oven allowing free circulation of air and thereby even heating of the load.
- Do not pack the load tightly since air is a poor conductor of heat.
- Switch on the power supply and control the temperature of the oven by adjusting the thermostat.
- Note the time when the desired temperature is reached (heating-up time).
- Hold the load in the oven at this temperature for a definite period of time (holding period). This is usually 60 minutes at 160°C.
- Do not overheat since it would char the cotton plugs and paper wrappings.

Autoclaves and hot air ovens can be used for disinfection of infectious waste before it is discarded. In addition, waste can be disposed of by boiling in detergent or by burial.

Boiling in detergent

In the absence of an autoclave, most specimen containers can be boiled in water having detergents to decontaminate. This process kills the vegetative bacteria but fails to destroy the spores and certain viruses. The easiest way to get best results is to add washing powder or sodium carbonate crystals, 60 grams to one litre of water in a big container and boil specimen containers in it for a minimum of 30 minutes.

Disinfection

Disinfection can be undertaken either chemically or by boiling. Boiling is an effective method to disinfect equipment e.g. needles and syringes, if autoclaving facilities are not available. Equipment which has already been cleaned should be boiled for 20 minutes. Chemical disinfection is used for heat-sensitive equipment that is damaged at high temperatures. Commonly-used chemical disinfectants include chlorine releasing compounds; ethyl and isopropyl alcohol, quaternary ammonium compounds and glutaraldehyde.

The synopsis of a few commonly-used disinfectants is given in Table 1.

Preferred methods of sterilization for common articles are given in Table 2.

Decontamination of some of the commonly reusable equipment has been briefly presented in Table 3.

Table 1: **Disinfectants and their mode of application***

Target	Disinfectant	Strength to use (disinfectant/ material V/V)	Application	Time of exposure
Skin	Ethanol	70%	Direct	2 minutes
	Iodine	1%	Direct	2 minutes
	Povidone iodine	1%	Direct	2 minutes
	Quaternary ammonium comp		Direct	2 minutes
Blood	Cresol (pH 9)	5%	2:1	6 hours
	Ca hypochlorite	1%	2:1	6 hours
Urine	Cresol (pH 9)	5%	1:1	4 hours
Sputum	Cresol (pH 9)	5%	1:1	4 hours
Faeces	Cresol (pH 9)	5%	2:1	6 hours
	Hypochlorite (Na/Ca)	1%	3:1	6 hours
	Ca hydroxide	20%	2:1	6 hours
Work benches	Lysol	5%	Direct	4 hours
	Cresol	1%	Direct	4 hours
	Hypochlorite	5%	Direct	4 hours
	Chloramine-T		Direct	4 hours
Glassware	Hypochlorite	1%	Direct	4 hours
Lab instruments	Hypochlorite	0.1%	Direct	4 hours
	Isopropanol	70%	Direct	4 hours

* Based upon: Basics of quality assurance: WHO/EMRO, 1992, page 162

Table 2: **Preferred methods of sterilization for common-use articles**

Autoclaving
Animal cages
Sugar tubes
Lab. coats
Cotton
Filters
Instruments
Culture media

Rubber
Gloves, stopper, tubing

Glass
Slides, syringes, test tubes
Enamel metal trays
Wire baskets

Hot air oven
Glass ware
Beakers
Flasks
Petridish
Pipette
Slides
Glass syringes
Test tubes
Powders

Wood
Tongue depressor, applicator

*Table 3: **Disinfection of specific equipment***

Container/material	Method of choice for decontamination	Alternative method of decontamination
Reusable stool container	Autoclaving 121°C for 30 minutes	Fill the jar having stool with 5% solution of phenol and keep for 24hours
	Empty into lavatory*	Empty into lavatory*
Reusable containers of CSF, pus, sputum	Autoclaving	Boiling in detergent
Urine bottles (after emptying in lavatory*)	Autoclaving	Fill with 2% phenol or 1% bleach, leave for 4 hours, clean with detergent
Blood containers	Autoclaving	Soak overnight in strong disinfectant(5% cresol; 1% Ca hypochlorite, 1:2 V/V)
Glass microscope slides**	Autoclaving	Soak overnight in 5% phenol

* If the lavatory is connected to a septic tank, phenol or other antiseptics should not be put into the lavatory.

** Glass microscope slides which have been used for the diagnosis of tuberculosis should be discarded after keeping them soaked in detergent overnight.

Biohazard waste management

Waste is defined as any solid, liquid or gaseous material that is no longer used and will either be recycled, disposed of or stored in anticipation of treatment and/or disposal.

Storage

Prior to disposal, all biohazardous waste should be maintained and stored separately from the general waste stream and from other hazardous wastes. The containers used to store biohazardous waste should be leak-proof, clearly labelled with a red or orange universal biohazard symbol and sealed tightly when transported. In certain cases, it may be necessary to double-bag the waste to prevent leakage. Any biohazardous sharps, such as infectious needles and scalpels, must be placed in containers that are puncture-resistant, leak-proof on all sides and the bottom, and close-able. These containers can then be placed in a standard biohazard bag.

Disposal options

There are three main disposal options:

- render the waste noninfectious by autoclaving and dispose it in the general waste stream. If autoclaving is not possible, decontaminate with chemical disinfectants or by boiling for 20 minutes before disposal.
- on-site incineration, if possible.
- transportation of locally-generated waste to a distant appropriate facility.

Incineration is the preferred disposal option. Not only does this method render the waste noninfectious but it also changes the form and shape of the waste. Sterilization is an effective method for decontaminating waste, but it does not alter the appearance of the waste. Steam sterilization in an autoclave at a temperature of 121^{o}C for at least 15 minutes destroys all forms of microbial life, including high numbers of bacterial spores. This type of complete sterilization can also be accomplished using dry heat which requires a temperature of 160-170^{o}C for 2-4 hours. However, it must be ensured that heat comes in contact with the material to be rendered sterile. Therefore, bottles containing liquid material should have loosened caps or cotton plug caps to allow for steam and heat exchange within the bottle. Biohazard bags containing waste should be tied loosely. Once sterilized, biohazardous waste should be sealed in appropriate containers, labelled as disinfected waste and disposed of in an approved facility.

Biological waste should be clearly labelled prior to disposal and complete records should be maintained.

Burial

It is not a decontaminating process *per se*. However, it does prevent the infectious material from becoming a reservoir of infection if properly buried. It requires digging a pit of almost 5 meters depth and 2 meters width and having a tightly fitted heavy lid on top. Disposable containers with clinical material are thrown daily into it and the lid is replaced immediately after throwing the specimens. Once a week, the refuse is covered with a layer of quicklime. If quicklime is not available, the refuse is covered with almost 10 cm thick layer of dried leaves once a week.

Further reading

El-Nageh MM et al. Basics of Quality Assurance for Intermediate and Peripheral Laboratories. WHO Regional Publication, Eastern Mediterranean Series No 2, 156-166, 1992.

4. Staining Techniques

Staining of the clinical material or the bacteria from colonies on laboratory media provide a direct visualization of the morphology of the organisms as well as their reactions to the chemicals present in stains. This is an invaluable and easy-to-use tool for establishing the identity of various microorganisms. Some of the commonly-used staining techniques are:

- Methylene blue staining
- Gram staining
- Albert staining
- Ziehl Neelsen staining (Acid fast staining)
- India ink staining
- Iodine staining for ova and cysts in faeces

Methylene blue staining

Ingredients and preparation

Methylene blue	0.3 gm
Distilled water	100 ml

Dissolve the dye in water. Filter through a filter paper.

Staining procedure

- Make a smear on a glass slide, dry in air and fix by passing it over the flame of a burner 3-4 times.
- Stain for one minute by pouring methylene blue solution over the smear.
- Wash with water, blot dry and examine under the oil immersion of light microscope.

Uses

The stain is used to make out clearly the morphology of the organisms e.g. *Yersinia pestis* in exudate, *Haemophilus influenzae* in CSF and gonococci in urethral pus.

Gram staining

This is the most extensively used differential stain that divides bacteria into two major groups. Those which retain crystal violet dye after treatment with iodine and alcohol appear purple or bluish purple and are designated as Gram positive. Those bacteria which lose the crystal violet show the colour of the counter stain employed. The commonly-used counter stain is saffranin which gives a pink/red colour to bacteria and these organisms are labelled as Gram negative.

Ingredients and preparation

Crystal violet

Solution A

Crystal violet	2.0 gm
Ethanol, 95%	20 ml

Solution B

Ammonium oxalate	0.8 gm
Distilled water	80 ml

Mix solutions A and B. Store for 24 hours before use.

Gram iodine

Iodine crystals	1.0 gm
Potassium iodide	2.0 gm
Distilled water	300 ml

Grind the dry iodine and potassium iodide in a mortar. Add water, a few ml at a time, and grind thoroughly after each addition until the iodine and iodide dissolve. Rinse the solution into an amber glass bottle with the remainder of the distilled water.

Saffranin solution

Stock solution

Saffranin O	2.5 gm
Ethanol, 95%	100 ml

Working solution

Stock solution	10 ml
Distilled water	90 ml

Staining procedure

- Make a thin smear on a clean glass slide, dry it in air and fix by passing through flame of a burner.
- Cover the smear with crystal violet, keep for one minute.
- Wash the slide with water, then cover with Gram iodine and let it stand for one minute.
- Wash the slide with water.
- Decolour with acetone/alcohol, rocking the slide gently for 10-15 seconds till the violet colour comes off the slide.
- Wash with water immediately.
- Counterstain with saffranin. Let the counterstain stand for 30 seconds.
- Wash with water, blot dry and examine under the oil immersion lens of a microscope.

Uses

Widely used in diagnostic bacteriology mainly to differentiate organisms on the basis of morphology and Gram reaction.

Albert staining

Ingredients and preparations

Albert stain I

Toluidine blue	0.15 gm
Malachite green	0.20 gm
Glacial acetic acid	1.0 ml
Alcohol(95%)	2.0 ml
Distilled water	100 ml

Grind and dissolve the dyes in alcohol, add water and then add acetic acid. Let the mixture stand for 24 hours and then filter.

Albert stain II

Iodine	2.0 gm
Potassium iodide	3.0 gm
Distilled water	300 ml

Dissolve iodine and potassium iodide in water by grinding in a mortar with a pestle. Filter through a filter paper.

Staining procedure

- Cover the heat-fixed smear with Albert stain I. Let it stand for two minutes.
- Wash with water.
- Cover the smear with Albert stain II. Let it stand for two minutes.
- Wash with water, blot dry and examine.

Uses

To demonstrate metachromatic granules in *C.diphtheriae*. These granules appear bluish black whereas the body of bacilli appear green or bluish green.

India ink staining

Staining procedure

- Place a loopful of India ink on the side of a clean slide.
- A small portion of the solid culture is suspended in saline on the slide near the ink and then emulsified in the drop of ink, or else, mix a loopful of liquid culture of specimens like CSF with the ink.
- Place a clean cover slip over the preparation avoiding air bubbles.
- Press down, or blot gently with a filter paper strip to get a thin, even film.
- Examine under dry objectives followed by oil immersion.

Use

To demonstrate the capsule which is seen as an unstained halo around the organisms distributed in a black background. This is employed for fungal diagnostics especially for *Cryptococcus neoformans*.

Ziehl Neelsen staining

Ingredients and preparations

Carbol fuchsin	1%
Sulphuric acid	25%
Methylene blue	0.1%

- Select a new, unscratched slide and label the slide with a Laboratory Serial number.
- Make a smear from yellow purulent portion of the sputum using a bamboo stick. A good smear is spread evenly, 2 cms x 3 cms in size and is neither too thick nor too thin. The optimum thickness of the smear can be assessed by placing the smear on a printed matter, the print should be readable through the smear.

- Let the smear air-dry for 15-30 minutes.
- Fix the smear by passing the slide over the flame 3-5 times for 3-4 seconds each time.
- Place the fixed slide on the staining rack with the smeared side facing upwards.
- Pour filtered 1% carbol fuchsin over the slide so as to cover the entire slide.
- Heat the slide underneath until vapours start rising. Do not let carbol fuchsin to boil or the slide to dry. Continue the process up to five minutes.
- Allow the slide to cool for 5-7 minutes.
- Gently rinse the slide with tap water to remove the excess carbol fuchsin stain. At this point, the smear on the slide looks red in colour.
- Decolor the stained slide by pouring 25% sulphuric acid on the slide and leaving the acid for 2-4 minutes.
- Lightly wash away the free stain. Tip the slide to drain off the water.
- If the slide is still red, reapply sulphuric acid for 1-3 minutes and rinse gently with tap water.
- Counter stain the slide by pouring 0.1% methylene blue solution onto the slide and let it stand for one minute.
- Gently rinse the slide with tap water and tip the slide to drain off the water.
- Place the slide in the slide tray and allow it to dry.
- Examine the slide under a microscope using 40 x lens to select the suitable area of the slide and examine under 100 x lens using a drop of immersion oil.

Uses

Distinguishes acid fast bacilli such as *Mycobacterium tuberculosis* and *M.leprae* from other non-acid fast bacilli.

Iodine staining for ova and cysts

- On a clean glass slide place one drop of normal saline and one drop of 2% iodine solution at two different sites.
- Mix a portion of stool first with normal saline and then with iodine solution with the help of a wire loop or applicator.
- Place coverslips on both the emulsions.
- Examine the preparations under 10x and 40x of the microscope for various ova and cysts.

Quality control of stains

Test all stains at appropriate intervals for their ability to distinguish positive and negative organisms and document the results. The performance standards for Ziehl-Neelsen and Gram staining are as given below:

Stain	Control organism/ material	ATCC No*	Expected result
Ziehl-Neelsen	*Mycobacterium spp.* *E. coli*	25177 25922	Pink red bacilli Blue bacilli
Gram	*E. coli* *S. aureus*	25922 25923	Gram -ve bacilli Gram +ve cocci
Iodine solution	Formalin treated stool specimen with cysts		Visible cyst nuclei

* If no standard strains are available, known laboratory strains should be used as controls.

The quality control procedure for stains needs to be performed on a weekly basis and also as and when a new lot of reagents for staining is procured/prepared.

Further reading

1. Manual of basic techniques for a health laboratory, WHO, 1980.
2. Bailey & Scott's Diagnostic Microbiology by Baron, Peterson and Finegold, 9th Ed, Mosby, 1994.

5. Bacteriological Media

The role of suitable quality culture media for cultivation of microorganisms cannot be over emphasised. On it depends the very success of isolation of aetiological agents. Only in exceptional cases, can an organism be identified on the basis of its morphological characteristics alone.

Types of media

Bacteriological media can be broadly sub-divided into four categories.

(1) Ordinary culture media

These are routinely employed in a laboratory e.g. nutrient broth, nutrient agar, infusion broth and lysate media.

(2) Enriched media

Certain organisms do not grow on ordinary nutrient media. They require growth-promoting ingredients such as blood, glucose, serum, egg, etc. The media containing ingredients which enhance their growth-promoting qualities are enriched media e.g. blood agar, chocolate agar and Loeffler medium.

(3) Enrichment media

Enrichment media are liquid media containing chemical constituents which inhibit some normal flora and allow pathogens which may be present in very small number in the specimen, to grow unhampered and thus enriching them. Isolated colonies of these organisms may be obtained by subculturing onto solid media. An example of enrichment media is selenite broth used for primary isolation of enteric bacteria.

(4) Differential and selective media

Differential media have got some chemical constituents which characterize different bacteria by their special colonial appearances in the culture e.g. MacConkey agar contains lactose as a substrate and neutral red as an indicator. Bacteria fermenting lactose produce acid and this will change the colour of the indicator and thus the colonies will turn red. The red lactose fermenting colonies can be differentiated from the pale non-lactose fermenting colonies.

Selective media will selectively permit the growth of pathogens and inhibit the commensals. In addition, it may differentiate the pathogen from commensals that grow by the colour and opacity of the colonies e.g. blood tellurite medium for *C.diphtheriae*.

In addition, transport media are also frequently used to sustain the viability of organisms when a clinical specimen is to be transported from the periphery to laboratory. The transport medium prevents the outgrowth of contaminants during transit and sustains the pathogen. Cary and Blair and Stuart media are two examples of this group of media.

Preparation of media and checking of pH

Presently, a wide range of culture media are available commercially in the form of dehydrated media. These media are simply reconstituted by weighing the required quantities and by adding distilled water, as per the manufacturer's instructions.

The pH determination can be conveniently done with the use of Lovibond comparator with phenol red indicator disc.

- ➢ Take two clean test tubes and add 5 ml of the medium to each of the tubes. One serves as a blank while phenol red indicator is added to the other tube.
- ➢ Compare the colour of the medium with the phenol red indicator at the appropriate pH marking.
- ➢ Add N/10 NaOH or N/10 HCl, drop by drop till the colour of the medium matches the colour of the disc at the required pH reading.
- ➢ Calculate the volume of the NaOH or HCL of 1/10 strength for 5 ml of the medium to get the required pH.
- ➢ Based on the calculation, the volume of 1N NaOH or IN HCl required for the total volume of medium can be calculated and added.
- ➢ Check the pH of the medium once again before use.

The quantity of agar given in the formulae of media may have to be changed depending upon the quality of agar used. The concentration varies from batch to batch and should be such that will produce a sufficiently firm surface on solidification. This can be tested by streaking with inoculating wire.

In some laboratories media are prepared by individual measurement of ingredients and then mixing the same. Hence the method of preparation is given likewise:

Nutrient broth

Meat extract	10.0 gm
Peptone	10.0 gm
Sodium chloride	5.0 gm
Distilled water	1000 ml

Mix the ingredients and dissolve them by heating in a steamer. When cool, adjust the pH to 7.5-7.6.

Nutrient agar

To the ingredients as in nutrient broth, add 15 gm agar per litre. Dissolve the agar in nutrient broth and sterilize by autoclaving at 121°C for 15 minutes. Prepare plates and slopes as required.

Glucose broth

Nutrient broth	900 ml
Glucose (10% solution)	100 ml

- ➢ Dissolve 9 gm glucose in distilled water and sterilize by tyndallisation.
- ➢ Add l00 ml of the glucose solution to 900 ml of sterile nutrient broth.
- ➢ Dispense 60 ml each in 100 ml pre-sterilized culture bottles.
- ➢ Sterilize by open steaming at l00°C for one hour.

Blood agar

Nutrient agar	100 ml
Sheep blood (defibrinated)	10 ml

- ➢ Melt the sterile nutrient agar by steaming, cool to 45°C.
- ➢ Add required amount of sheep blood aseptically with constant shaking.
- ➢ Mix the blood with molten nutrient agar thoroughly but gently, avoiding froth formation.
- ➢ Immediately pour into petri dishes or test tubes and allow to set.

Chocolate agar

The ingredients are essentially the same as in blood agar.

- ➢ Melt the sterile nutrient agar by steaming and cool to about 75°C.
- ➢ Add blood to the molten nutrient agar and allow to remain at 75°C after gently mixing till it is chocolate brown in colour.
- ➢ Pour in petri dishes or test tubes for slopes as desired.

XLD agar

Xylose	3.5 gm
1 – lysine	5.0 gm
Lactose	7.5 gm
Sucrose	7.5 gm

Sodium chloride	5.0 gm
Yeast extract	3.0 gm
Sodium desoxycholate	2.5 gm
Sodium thiosulphate	6.8 gm
Ferric ammonium citrate	0.8 gm
Phenol red	0.08 gm
Agar agar	15.0 gm
Water	1000 ml

Weigh the ingredients into a flask and add distilled water. Mix the contents well and steam it for 15 minutes (do not autoclave). Cool to 56°C and pour in plates.

Buffered glycerol saline

Glycerol	300 ml
Sodium chloride	4.2 gm
Disodium hydrogen phosphate	10.0 gm
Na_2 H PO_4 Anhydrous	15.0 gm
Phenol red aqueous solution 0.02 per cent	15.0 ml
Water	700 ml

- ➢ Dissolve NaCl in water and add glycerol.
- ➢ Add disodium hydrogen phosphate to dissolve.
- ➢ Add phenol red and adjust pH to 8.4.
- ➢ Distribute 6 ml in universal containers (screw -capped bottles of 30 ml capacity). Autoclave at 115°C for 15 minutes.

Loeffler serum medium

Nutrient broth	100 ml
Serum (sheep or horse or ox)	300 ml
Glucose	1.0 gm

- ➢ Dissolve glucose in nutrient broth and sterilize at 121°C for 15 minutes.
- ➢ Add serum aseptically.
- ➢ Mix thoroughly but gently, avoiding froth formation.
- ➢ Distribute in sterile test tubes or quarter ounce screw-cap bottles.
- ➢ Inspissate the medium in a slanting position in a water inspissator at 82°C for two hours.
- ➢ In the absence of an inspissator, the medium may be coagulated by standing over the top of a steam sterilizer for 6-7 minutes.

Blood tellurite agar

Agar base

Meat extract	5.0 gm
Peptone	10.0 gm
Sodium chloride	5.0 gm
Agar	25.0 gm
Water	1000 ml

Dissolve the ingredients and adjust the pH to 7.6. Distribute in 100 ml quantities in a bottle and autoclave at 121°C for 15 minutes.

Glycerolated blood tellurite mixture

Sterile defibrinated sheep blood	14 ml
Sterile glycerol	6 ml
Sterile potassium tellurite solution (1% in water)	4 ml

Sterilize the glycerol in hot air oven at 160°C for 60 minutes and the tellurite solution by autoclaving at 115°C for 20 minutes. Mix the ingredients in a sterile flask, incubate for 1-2 hrs. at 37°C, then refrigerate. Haemolysis is complete after 24 hrs. The mixture keeps well in a refrigerator. One per cent solution of good quality tellurite is sufficient but 2% of some batches may be required.

Preparation of complete medium

Glycerolated blood tellurite mixture	24 ml
Agar base	100 ml

Melt the agar, cool to 45°C, add blood and tellurite and pour in sterile petri dishes.

Salt broth (10%)

Meat extract	10.0 gm
Peptone	10.0 gm
Sodium chloride	100.0 gm
Distilled water	1000 ml

Prepare as for nutrient broth, distribute and sterilize at 121°C for 15 minutes.

Peptone water

Peptone	10.0 gm
Sodium chloride	5.0 gm
Distilled water	1000 ml

Dissolve peptone and sodium chloride in distilled water by heating. Adjust pH to 7.2. Distribute in test tubes and sterilize by autoclaving at 121°C for 15 minutes.

Alkaline peptone water

Prepare peptone water as described above. Adjust pH of peptone water (7.2 to 9.2). Distribute in test tubes and sterilize at 121°C for 15 minutes.

MacConkey agar

Sodium taurocholate	5.0 gm
Peptone	20.0 gm
Sodium chloride	5.0 gm
Lactose	10.0 gm
Agar	15.0 gm
Distilled water	1000 ml
Neutral red (2% solution in 50% ethanol)	3.5 ml

- Mix 5 gm sodium taurocholate or bile salts, 20 gm of peptone, 5 gm sodium chloride and 15 gm agar with 1000 ml water.
- Steam until the solids are dissolved.
- Cool to about 50°C, and at this temperature adjust reaction to pH 7.5 to 7.8. Autoclave at 121°C for 15 minutes and filter while hot through a good grade of filter paper, or a plug of cotton wrapped in gauze placed in the funnel.
- Adjust reaction of the filtrate to pH 7.3 at 50°C or pH 7.5 at room temperature. Add 10 gm lactose and 3.5 ml of 2% solution of neutral red in 50% ethanol.
- Mix thoroughly, distribute in flasks and sterilize in the autoclave at 121°C for 15 minutes.
- For use, melt in the steamer, pour into sterile petri dishes and allow to set.

Bile salt agar

Peptone	10.0 gm
Meat extract	5.0 gm
Sodium taurocholate	5.0 gm
Sodium chloride	5.0 gm
Agar	15.0 gm
Distilled water	1000 ml

- Dissolve by steaming in 1000 ml of water, 10 gm peptone, 5 gm meat extract, 5 gm sodium chloride, 15 gm agar and 5 gm sodium taurocholate.
- Adjust pH to 8.5 with sodium hydroxide solution.
- Cool and filter through a good grade filter paper or absorbent cotton wool previously wetted with water.
- Distribute into sterile flasks in convenient amounts and sterilize by autoclaving at 121°C for 15 minutes.
- Plates are made by melting the stock medium and pouring into sterile petri dishes.

Agar base

Meat extract	20.0 gm
Peptone (proteose)	20.0 gm
Agar	90.0 gm
Lactose	40.0 gm
Neutral red (2% solution in 50% ethanol)	5.0 gm
Distilled water	4.0 litre

Solution A

Sodium citrate, $2H_2O$	17.0 gm
Sodium thiosulphate	17.0 gm
Ferric ammonium citrate	2.0 gm
Distilled water	100.0 ml

Solution B

Sodium desoxycholate	10.0 gm
Distilled water	100 ml

Agar base

- Dissolve by heating 20 gm meat extract in 200 ml water and make the solution just alkaline with 50 per cent sodium hydroxide solution.
- Boil and filter through filter paper.
- Adjust to pH 7.3, make upto 200 ml and add 20 gm proteose peptone.
- In another vessel, dissolve 90 gm agar in 3700 ml water by steaming.
- Filter the agar solution, add the meat extract peptone solution to it and mix.
- Add 5 ml neutral red solution in 50% ethanol and 40 gm lactose.
- Make up to 4 litres with water.
- Mix, bottle accurately in lots of 100 ml, and sterilize in an autoclave by free steaming for one hour and then at 121°C for 10 minutes.

Solution A

Dissolve 17 gm sodium citrate, 17 gm sodium thiosulphate and 2 gm ferric ammonium citrate (green scales) in 100 ml of sterile distilled water with heating.

Solution B

Dissolve 10 gm sodium desoxycholate in 100 ml of sterile distilled water.

- ➢ Sterilize solution A and B at 60°C in water bath for one hour.
- ➢ For preparing desoxycholate citrate agar medium, melt 100 ml of the agar base in a water bath and add 5 ml each of the solutions A and B in the order given, using separate pipettes.
- ➢ Mix well after each addition.
- ➢ Cool the tube at 50 to 55°C.
- ➢ Pour into sterilized petri dishes and allow to set.
- ➢ Dry the surface of the medium in the incubator before use.

Selenite F broth

Sodium hydrogen selenite	4.0 gm
Peptone	5.0 gm
Lactose	4.0 gm
Disodium hydrogen phosphate ($Na_2HPO_4,12H_2O$)	9.5 gm
Sodium dihydrogen phosphate	0.5 gm
Distilled water	1000 ml

- ➢ Dissolve 4 gm sodium hydrogen selenite, 5 gm peptone, 4 gm lactose, 9.5 gm disodium hydrogen phosphate and 0.5 gm sodium dihydrogen phosphate in 1000 ml of sterile water (water autoclaved at 121°C for 30 minutes) with sterile precautions and distribute the yellowish solution in 10 ml amounts in sterile screw-capped bottles.
- ➢ Steam at 100°C for 30 minutes. The medium should not be autoclaved.
- ➢ There may be a slight red precipitate in the medium, but this does not interfere with the action of the medium.
- ➢ The pH of the medium as prepared should be 7.1 and the quantity of phosphate added may be varied slightly to achieve this.

Media for carbohydrate fermentation

(a)	Peptone	10.0 gm
	Sodium chloride	5.0 gm
	Distilled water	900 ml
(b)	Carbohydrate base	10.0 gm
	Distilled water	90 ml

(c) Andrade's indicator 10 ml

- ➢ Dissolve 10 gm peptone and 5 gm sodium chloride in 900 ml water.
- ➢ Adjust pH to 7.0 to 7.3 so that after addition of 10 ml of Andrade's indicator the pH should be 7.5.
- ➢ Sterilize at 121°C for 15 minutes.
- ➢ Dissolve 10 gm of the requisite sugar in 90 ml water and steam for 30 minutes or sterilize by filtration.
- ➢ With sterile precautions, add 90 ml of this sugar solution and 10 ml of Andrade's indicator solution to 900 ml of the sterile peptone water solution. Distribute into sterile test tubes containing inverted Durham fermentation tubes. Steam for 30 minutes.
- ➢ Prepare Andrade's indicator solution by adding 1 N sodium hydroxide solution to 0.5% aqueous solution of acid fuchsin until the colour of the indicator solution is just yellow.

Lowenstein-Jensen medium

Salt solution

Potassium dihydrogen phosphate (KH_2PO4)	2.40 gm
Magnesium sulphate ($MgSO_4.7H_2O$)	0.24 gm
Magnesium citrate	0.60 gm
Asparagine L(Pure)	3.60 gm
Glycerol	12.0 ml
Distilled water	600 ml
Whole egg	1000 ml
Malachite green 2%	20 ml

Dissolve the salts and glycerol in water over a water bath. Autoclave for 15 minutes at 121°C, cool to room temperature.

Beaten egg

- ➢ Use only fresh eggs preferably not more than three days old.
- ➢ Using a soft brush and soap and with soda solution clean the outside of the egg's wall. Leave the eggs in 5% soap and soda solution for 30 minutes.
- ➢ Place in running tap water till the water is perfectly clear. Drain and dry the eggs.
- ➢ Just before breaking, clean the outside of the eggs with a piece of sterile gauze dipped in alcohol.
- ➢ Break the eggs into a sterile flask aseptically.
- ➢ Close with a rubber stopper and shake vigorously until well homogenized. Filter through two or three layers of sterile gauze stretched over a sterile funnel and collect the filtrate in a sterile container.

Malachite green solution

- Dissolve I gm malachite green in 50 ml distilled water in a screw capped bottle and sterilise at 121°C for 15 minutes.
- Add the eggs to the salt solution aseptically and mix well.
- Add malachite green and mix.
- Pour into a sterile aspirator jar and let it stand for 30 minutes for the air bubbles to escape.
- Fill into tubes or bottles.
- Inspissate for 40 minutes at 82°C to 85°C.
- While inspissating, the bottle should be kept flat but the tubes have to be sloped.

Cary and Blair transport medium

Sodium thioglycollate	1.5 gm
Disodium phosphate	1.1 gm
Sodium chloride	5.0 gm
Agar	5.0 gm
Calcium chloride, (1% solution)	9.0 ml
Distilled water	1000 ml

- Dissolve by heating 1.5 gm sodium thioglycollate, 1.1 gm disodium phosphate, 5 gm sodium chloride and 5 gm agar in 990 ml distilled water.
- Cool to 50°C and add 9 ml of one per cent aqueous solution of calcium chloride. Adjust the pH to 8.4.
- Dispense in 7 to 10 ml amounts in wide mouth screw capped bottles.
- Steam for 15 minutes, cool and tighten the caps.

Stuart transport medium

Thioglycolic acid	2 ml
Sodium hydroxide, (IN NaOH)	12-15 ml
Sodium glycerophosphate, 20% aqueous	100 ml
Calcium chloride, $CaCl_2$, 1% aqueous	20 ml
Distilled water	900 ml

Mix the ingredients, adjust pH to 7.2 with IN sodium hydroxide solution.

Agar solution

Agar	6.0 gm
Distilled water	1000 ml
Dissolve by steaming	

Preparation of complete medium

Anaerobic salt solution	900 ml
Agar solution	11 ml
Methylene blue, 0.1% aqueous	4 ml

- Melt the agar and add the salt solution.
- Adjust the pH to 7.3-7.4.
- Add the methylene blue and distribute in small bottles filling nearly to capacity. Autoclave at 121°C for 15 min. and immediately tighten caps.
- When cool, the medium should be colourless.

Venkataraman-Ramakrishnan (VR) holding medium

- Dissolve 12.4 gm. boric acid and 14.9 gm potassium chloride in 800 ml hot distilled water, cool and make up to 1000 ml with distilled water.
- To 250 ml of this stock solution, add 133.5 ml N/5 sodium hydroxide solution. Make up to 1000 ml with distilled water and add 20 gm common salt. Shake and dissolve.
- Filter through filter paper and dispense in 10 to 15 ml amounts in wide-mouthed screw capped bottles.

A simple modification is as follows:

- Dissolve 20 gm of crude sea salt and 5 gm of peptone in distilled water to make one litre.
- Adjust the pH to 8.6 to 8.8.
- Dispense 10-15 ml amounts in wide-mouthed bottles with screw-caps.
- About 1-3 ml of stool specimen should be inoculated into the medium.

MacConkey broth for bacteriological examination of water

This medium is used for detecting the presence of coliform organisms in water.

Single strength

Sodium taurocholate (commercial)	5 gm
Peptone (any good make)	20 gm
Sodium chloride (NaCl)	5 gm
Lactose	10 gm
Bromocresol purple, 1%, solution in ethanol	5 ml
or Neutral red, 1% aqueous solution	5 ml
Water	1000 ml

- Dissolve the bile salt, peptone and sodium chloride.
- Steam for two hours, cool and transfer to the refrigerator overnight.
- Add lactose and when dissolved, filter cold through filter paper.
- Adjust the reaction to pH 7.4.
- Add indicator.
- Distribute in 5 ml amounts in 1-oz. bottles or 15 cm x 1.5 cm test tubes with Durham tubes.
- Autoclave at 121°C for 15 minutes.

Double strength

- Prepare as above, but with half the amount of water.
- Distribute in 50 ml amounts in 5-oz bottles using 3x3/8 inch test tubes with Durham tubes, and in 10 ml amounts in 1-oz bottles using 2X0.25 inch Durham tubes.

Sabouraud Dextrose Agar (SDA)

Ingredients

	Original SDA	Emmon's modification
Dextrose Peptone	40 gm	20 gm
Neopeptone	10 gm	10 gm
Agar	20 gm	20 gm
Distilled water	1000 ml	1000 ml
Adjust final pH	5.6	6.8 to 7.0

Preparation

Mix the ingredients in water by heating. Adjust the pH. Sterilize in the autoclave at 121°C for 10 minutes.

10% Potassium Hydroxide

Ingredients and preparation

Potassium hydroxide	10 gm
Distilled water	100 ml

Dissolve 10 grams of potassium hydroxide in 100 ml of distilled water.

Lactophenol cotton blue mounting medium

Ingredients and preparation

Phenol crystals	20 gm
Lactic acid	20 gm
Glycerol	40 gm
Cotton blue (poirrier's blue)	0.05 gm
Distilled water	20 ml

Melt the phenol crystals in water bath. Mix water and phenol crystals. Add 0.05 gram of cotton blue into a mortar and grind it with a pestle. Add water and phenol little by little and make it a nice paste. Add the remaining amount of water and phenol, mix well. Then add lactic acid and glycerol and mix well. Filter through a filter paper.

Desoxycholate Citrate Agar (DCA)

Ingredients

Proteose peptone, No.3	10.0 gm
Lactose	10.0 gm
Sodium citrate	20.0 gm
Ferric ammonium citrate	2.0 gm
Sodium desoxycholate	5.0 gm
Bacto agar	15.0 gm
Neutral red (1% solution)	2.3 ml
Beef infusion broth	1000 ml
pH 7.4	

Preparation

Add Proteose peptone, sodium citrate, ferric ammonium citrate and bacto agar to the beef Infusion broth, pH of which has already been adjusted. Recheck pH. Keep it in a water bath for 30 minutes for dissolving. Then add lactose, sodium desoxycholate and neutral red solution and mix. Then pour in plates.

Bismuth Sulfite Agar (BSA) (Wilson & Blair medium)

Ingredients

Polypeptone peptone	10.0 gm
Beef extract	5.0 gm
Dextrose	5.0 gm
Disodium phosphate	4.0 gm
Ferrous sulfate	0.3 gm
Bismuth sulfite indicator	8.0 gm
Brilliant green	0.025 gm
Agar	20.0 gm
Distilled water	1000 ml
pH 7.5 $\pm$ 0.2	

Preparation

Mix the ingredients in distilled water. Allow to stand for five minutes and mix thoroughly. When a uniform suspension has been obtained, heat with frequent agitation and boil for one minute. Cool to about 50°C. Mix well and pour into sterile plates.

Mueller-Hinton Agar (MHA)

Ingredients

Beef extract	2.0 gm
Acidicase Peptone	17.5 gm
Starch	1.5 gm
Agar	17.0 gm
Distilled water	1000 ml

Final pH 7.4 $\pm$ 0.2

Preparation

Dissolve the ingredients in one liter of distilled water. Mix thoroughly. Heat with frequent agitation and boil for one minute. Dispense and sterilize by autoclaving at 121°C for 15 minutes. Do not overheat. When remelting the sterile medium, heat as briefly as possible.

Saponin lysed blood agar plus VCNT (A) inhibitors

A selective medium for the growth of *N.gonorrhoeae* using GC agar base

GC agar base (Difco)	36 gm
Saponin lysed horse/sheep blood	90 ml (9% final concentration)
Distilled water	1000 ml
VCN inhibitor (10 ml vial)	1 vial
Trimethoprim (100 mg/L stock)	2 ml
(Amphotericin (1000 mg/L stock)	1 ml (optional))

Method

The GC agar base medium is prepared at single strength which is half the strength recommended by the manufacturer, since the addition of equal volume of 2% haemoglobin is replaced by adding lysed blood. Suspend 36 gm GC agar base in one litre of distilled water. Mix well, then steam or boil gently until it dissolves completely. Sterilize by autoclaving at 15 psi (121°C) for 15 minutes. Cool to 52-54°C in a waterbath. When the molten GC agar medium has cooled to this temperature, aseptically add the saponin lysed horse or sheep blood. VCN inhibitor solution, trimethoprim (and amphotericin if used). Mix well and pour 25 ml volumes in 90 mm diameter petri dishes. Allow the agar to set, then store the plates in an inverted position in the laboratory refrigerator (2-8°C) until required.

Performance of plated media

Samples of plates from each batch are selected for performance-testing and are inoculated with the appropriate stock cultures. For each type of medium, at least two or three microorganisms having growth characteristics with 'positive' and 'negative' results for the medium should be used. The size of inoculum and method of inoculating the test plates

must be standardized as closely as possible. In general, control organisms should be selected from an actively growing broth culture and a standard loopful of culture seeded directly onto the test medium, which is then streaked so as to obtain isolated colonies. After appropriate incubation, the results of the performance test are recorded. The medium is released for use in the clinical laboratory only if the results indicate satisfactory performance. In initiating a quality control programme, one must establish some priorities, such as beginning by testing those media that are most likely to demonstrate deficiencies. Top priority should be given to blood agar, chocolate agar and Thayer Martin agar media. Secondary priority should be accorded to selective enteric media such as MacConkey agar, XLD and bile salt agars.

A quantitative approach may be more useful for testing of performance of selective or inhibitory media such as Thayer Martin agar. *N gonorrhoeae* and *N.meningitidis* usually grow on Thayer Martin agar when the inoculum is heavy, but when a fairly light inoculum is used, the pathogens might be inhibited. Consequently, a somewhat quantitative performance test could detect deficiencies that would be overlooked if one simply inoculated test plates with undiluted stock cultures.

Further reading

Balows A, Hausler WJ, Herrman KL et al. Manual of Clinical Microbiology 5th Ed, American Soceity of Microbiologists, Washington, 1991.

6. Cultivation of Bacteria on Laboratory Media

Inoculation of Culture Media

For microbiological investigations it is essential to learn the skills of inoculating specimens onto culture media and subculturing from one medium to another.

Instrument for seeding media

This is selected according to the nature of the medium and inoculum. Platinum or nichrome wires of different gauges are used. Nichrome has oxidizing properties and hence in some of the tests where this property of bacterium is to be tested (e.g. oxidase test), platinum wire, instead of nichrome should be used. This wire is sterilized by holding it vertically in the flame of the burner so that the whole length of wire becomes red hot. It is allowed to cool down before it touches any material suspected to be having bacteria to avoid the heat killing the organisms. Presterilized disposable loops are now available commercially. The wire can be used as a:

- Straight wire to stab the culture, picking of single colonies as well as for inoculating the liquid media,
- Thick wire which is useful for lifting the viscid material such as sputum, and
- Wire loop which is usually of 2 mm diameter is most useful of all inoculating wires. These are preferred to seed a plate of medium as the straight wire usually cuts the agar.

Seeding a culture plate

There are three commonly employed techniques for seeding culture plates. The most common is shown in Fig. 1.

The inoculum from the clinical material or another plate is first spread out in the form of a primary inoculum (as at A in Fig 1) which is also called as 'well-inoculum' or only 'well'. The successive series of strokes B, C, D and E are made with the loop sterilized between each sequence. At each step the inoculum is derived from the most distal part of the immediately preceding strokes so as to gradually reduce the number of bacteria. This helps in obtaining isolated colonies.

In an alternative plating procedure one edge of a large loop is used to make a secondary well (see B in Fig 1). The other edge is then used to make succession of strokes across the remaining unseeded area.

When the inoculum is small or the medium is selective it can be more heavily inoculated (Fig 2). Several loop-fulls of the specimen are used to spread the primary inoculum (see A in Fig 2).

After sterilizing the loop, it is recharged by rubbing it over area A and the plate is seeded in parallel strokes.

Figure1: *Seeding a culture plate*

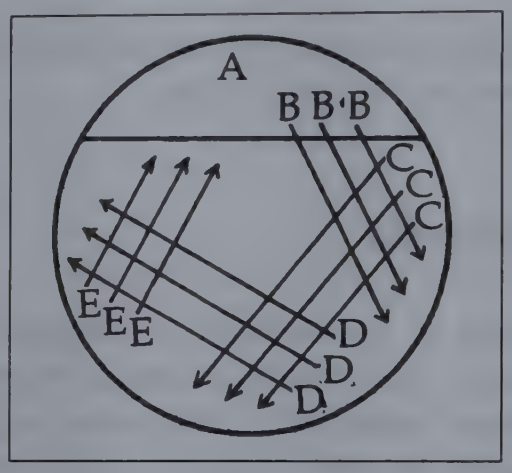
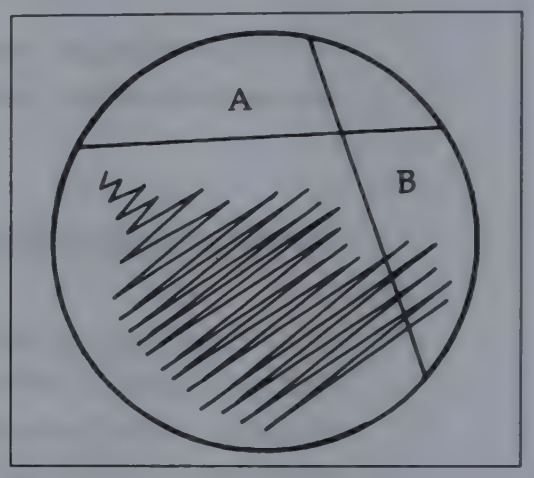


Figure 2: *Seeding with heavy inoculum*



Seeding a liquid medium

If the tubes have got cotton plugs, the mouth of the tubes should be heated in flame before and after any handling of tube to prevent contamination from the rims of tubes getting into the medium. It is not required when metal caps and screw-capped tubes are handled. Incline the tube containing the liquid medium to 45^{o} and deposit the inoculum on its wall above the surface of the liquid at its lower end. Return the tube to a vertical position. Now the inoculum shall be below the surface of the liquid.

Subculture from a solid medium to solid medium

Using a sterile wire or loop, a representative colony is touched and subcultured onto appropriate solid medium by touching the wire or loop onto the surface of the medium.

Important points about inoculation of culture media

- Aseptic technique is important to avoid contamination.
- When more than one medium is inoculated, follow a particular order. Inoculate media without inhibitors, followed by indicator and then selective media.
- While processing fluid specimen inoculate liquid media first to reduce the chances of carry over from contaminated solid media.
- Prepare smears for staining after all media have been inoculated.

- Properly label the media to be inoculated to avoid any mix-up of the specimens.
- Inoculate the media with clinical specimens as soon as possible.
- Minimize the aerosol production by opening the caps of liquid media slowly, avoiding vigorous shaking of the specimen and avoiding the expulsion of the last drop from the pipette.

Inoculation of carbohydrate fermentation media

These are inoculated as liquid media and incubated at 37°C for 18-24 hours. When the particular sugar is fermented, acid is produced which changes the pH of the medium thus turning phenol red into yellow. In case the fermentation is with the production of gas, a bubble of air is visible in Durham tube.

Seeding solid media in test tubes

Slopes of solid media are inoculated by streaking the surface of the agar with loop in a zig zag manner. Stab cultures are inoculated by plunging the wire into the centre of the medium.

Aerobic Incubation of cultures

For bacteria of medical importance, incubation is uniformally done at 37°C. Depending upon the workload a laboratory may have a tabletop incubator (suitable for peripheral laboratories) or a walk-in incubator. For prolonged incubations, as are required for the growth of *Mycobacterium tuberculosis,* screw-capped bottles should be used instead of petri dishes or tubes to prevent the drying of medium.

Incubation in an atmosphere with added carbon dioxide

Extra carbon dioxide is needed for optimal growth of organisms such as *Brucella abortus,* pneumococci and gonococci. The concentration of additional carbon dioxide needed is 5-10 per cent. The simplest method for having this environment is to put the plates in a container and generate CO_2 inside by lighting a candle in it just before putting on the lid. Pure CO_2 can also be introduced in a container. Carbon dioxide-generating kits are now available and so are incubators which can provide a predetermined and regulated amount of this gas. Special CO_2 incubators (also called capnoeic incubators) are available commercially.

Aseptic techniques

Aseptic techniques are important to protect the worker from infection from the clinical specimen and also to prevent contamination of the material under process. Aseptic conditions can be achieved by following steps:

- Open the caps and lids of the containers containing the specimen for the briefest period required.

- ➢ Do not keep the lids on the workbench.
- ➢ Inoculating loops should be put through the flame properly prior to introducing them into the specimen container.
- ➢ While working on the infectious material, keep the specimen away from the face.
- ➢ Loops should not contain fluid or large particles of matter that may splatter when placed in the flame.
- ➢ Avoid vigorous shaking of the specimen prior to opening; open the caps slowly to minimize aerosol production.
- ➢ Perform homogenization and grinding procedures involving tissue or biopsy specimen in safety cabinet.
- ➢ Keep all specimens, tubes and bottles of media in racks to reduce the risk of accidental spillage.
- ➢ Mop up the workbench clean with any disinfectant at the start and close of work.
- ➢ Wash hands with soap and water before and after handling infectious specimens.

Further reading

1. Isenberg HD(Ed) Clinical Microbiology Procedures handbook. American Society for Microbiology, Washington, DC, Vol 1, Section 1.4, 1992.
2. Collins CH, Lyne PM and Grange JM. Microbiological Methods. Butterworths, London, 94-96, 1995.
3. PHLS Standard Operating Procedures – Inoculation of culture media No B.SOP 54 Version:1, 1998.

7. Antimicrobial Susceptibility Testing

Antibiotic susceptibility testing has become a very essential step for the proper treatment of infectious diseases. It is used

- ➢ To guide the clinician in selecting the best antimicrobial agent.
- ➢ To accumulate epidemiological information on the resistance of microorganisms of public health importance.

The choice of drugs used in a routine antibiogram is governed by various considerations since only a few antimicrobial agents can be tested. Table 1 suggests the drugs to be tested in various situations. The drugs in Table 1 are divided into two sets. Set 1 includes drugs that are available in most hospitals and for which routine testing should be carried out for every strain. Tests for drugs in set 2 are to be performed only at the special request of the physician, or when the causative organism is resistant to the first-choice drugs, or when other reasons (allergy to a drug, or its unavailability) make further testing justified.

Table 1: ***Basic sets of drugs for routine susceptibility tests***

	Set 1	Set 2
Staphylococcus	Benzyl penicillin Oxacillin Erythromycin Tetracycline Chloramphenicol	Gentamicin Amikacin Co-trimoxazole Clindamycin
Intestinal	Ampicillin Chloramphenicol Co-trimoxazole Nalidixic acid Tetracycline	Norfloxacin
Enterobacteriaceae **Urinary**	Sulfonamide Trimethoprim Co-trimoxazole Ampicillin Nitrofurantoin Nalidixic acid Tetracycline	Norfloxacin Chloramphenicol Gentamicin
Blood and tissues	Ampicillin Chloramphenicol Cotrimoxazole Tetracycline Gentamicin	Cefuroxime Ceftriaxone Ciprofloxacin Piperacillin Amikacin
Pseudomonas aeruginosa	Piperacillin Gentamicin Tobramycin	Amikacin

Antimicrobial susceptibility tests measure the ability of an antibiotic or other antimicrobial agent to inhibit bacterial growth *in vitro*. This ability may be estimated by either the dilution method or the diffusion method. The recommended method for intermediate and peripheral laboratories is the modified Kirby-Bauer method, the methodology of which is given below:

Modified Kirby-Bauer method

Reagents

Mueller-Hinton agar

- Mueller-Hinton agar should be prepared from a dehydrated base according to the manufacturer's recommendations. The medium should be such that with standard strains, zone sizes within the acceptable limits are produced. It is important not to overheat the medium.
- Cool the medium to 45-50°C and pour into plates. Allow to set on a level surface, to a depth of approximately 4 mm. A 9 cm diameter plate requires approximately 25 ml of the medium.
- When the agar has solidified, dry the plates for immediate use for 10-30 minutes at 36°C by placing them in an upright position in the incubator with the lids tilted.
- Any unused plates may be stored in a plastic bag, which should be sealed and placed in a refrigerator. Plates stored in this way can be kept for two weeks.
- In order to ensure that the zone diameters are sufficiently reliable for testing susceptibility to sulfonamides and co-trimoxazole, the Mueller-Hinton agar must have low concentrations of the inhibitors thymidine and thymine. Each new lot of Mueller-Hinton agar should therefore be tested with a control strain of *Enterococcus faecalis* (ATCC 29212 or 33186) and a disc of co-trimoxazole. A satisfactory lot of medium will give a distinct inhibition zone of 20 mm or more that is essentially free of hazy growth or fine colonies.
- For testing the susceptibility of fastidious organisms, 5% blood should be added to the Mueller-Hinton agar base.

Antibiotic discs

Any commercially available discs with the proper diameter and potency can be used. Stocks of antibiotic discs should preferably be kept at -20°C, or the freezer compartment of a home refrigerator is convenient. A small working supply of discs can be kept in the refrigerator for up to one month. On removal from the refrigerator, the containers should be left at room temperature for about one hour to allow the temperature to equilibrate. This procedure reduces the amount of condensation that occurs when warm air reaches the cold container.

Turbidity standard

Prepare the turbidity standard by pouring 0.6 ml of a 1% (10 gm/L) solution of barium chloride dihydrate into a 100-ml graduated cylinder, and filling to 100 ml with 1% (10 ml/L) sulphuric acid. The turbidity standard solution should be placed in a tube identical to the one used for the broth sample. It can be stored in the dark at room temperature for six months, provided it is sealed to prevent evaporation.

Swabs

A supply of cotton wool swabs on wooden applicator sticks should be prepared. These can be sterilized in tins, culture tubes, or on paper, either in the autoclave or by dry heat.

Procedure

- To prepare the inoculum from the primary culture plate, touch with a loop the tops of each of 3-5 colonies, of similar appearance, of the organism to be tested.
- When the inoculum has to be made from a pure culture, a loopful of confluent growth is similarly suspended in saline. Inoculum from colonies of streptococci cannot be made by emulsification. Hence, with streptococci, after inoculation the culture tubes are incubated for 4-6 hours to get uniform turbidity which should be matched with the turbidity standards.
- Compare the tube with the turbidity standard and adjust the density of the test suspension to that of the standard by adding more bacteria or more sterile saline. Proper adjustment to the turbidity of the inoculum is essential to ensure that the resulting lawn of growth is confluent or almost confluent.
- Inoculate the plates by dipping a sterile swab into the inoculum. Remove excess inoculum by pressing and rotating the swabs firmly against the side of the tube above the level of the liquid.
- Streak the swab all over the surface of the medium three times, rotating the plate through an angle of 60° after each application. Finally, pass the swab round the edge of the agar surface. Leave the inoculum to dry for a few minutes at room temperature with the lid closed. The antibiotic discs may be placed on the inoculated plates using a pair of sterile forceps.
- A sterile needle tip may also be used to place the antibiotic discs on the plate. Alternatively, an antibiotic disc dispenser can be used to apply the discs to the inoculated plate.
- A maximum of seven discs can be placed on a 9-10 cm diameter plate. Six discs may be spaced evenly, approximately 15 mm from the edge of the plate, and one disc placed in the centre of the plate. Each disc should be pressed down gently to ensure even contact with the medium.
- The plates should be placed in an incubator at 35°C within 30 minutes of preparation. Temperatures above 35°C invalidate the results for oxacillin/ methicillin.
- Do not incubate in an atmosphere of carbon dioxide.

- After overnight incubation, the diameter of each zone(including the diameter of the disc) should be measured and recorded in mm. The results should then be interpreted according to the critical diameters by comparing with standard tables (Table 2).
- The measurements can be made with a ruler on the under surface of the plate without opening the lid.
- The end-point of inhibition is judged by the naked eye at the edge where the growth starts, but there are three exceptions:
  - With sulfonamides and co-trimoxazole, slight growth occurs within the inhibition zone; such growth should be ignored.
  - When β-lactamase producing staphylococci are tested against penicillin, zones of inhibition are produced with a heaped-up, clearly defined edge; these are readily recognizable when compared with the sensitive control, and regardless of the size of the zone of inhibition, they should be reported as resistant.
  - Certain *Proteus* spp. may swarm into the area of inhibition around some antibiotics, but the zone of inhibition is usually clearly outlined and the thin layer of swarming growth should be ignored.

Table 2:* ***Zone sizes with different antimicrobial agents***

Antimicrobial agent	When testing against	Disc content	Zone diameter nearest whole mm		
			Resistant	Intermediate	Susceptible
ß-LACTAMS					
Ampicillin	Enterobacteriaceae	10 µg	<13	14-16	>17
	Staphylococci	10 µg	<28		>29
	Enterococci	10 µg	<16		>17
Carbenicillin	Pseudomonas	100 µg	<13	14-16	>16
	Gram negatives	100 µg	<19	20-22	>23
Methicillin	Staphylococci	5 µg	<9	10-13	>14
Mezlocillin	Pseudomonas	75 µg	<15		>16
	Other Gram negatives	75 µg	<17	18-20	>21
Nafcillin	Staphylococci	1 µg	<10	11-12	>13
Oxacillin	Staphylococci	1 µg	<10	11-12	>13
Penicillin	Staphylococci	10 units	<28		>29
	Enterococci	10 units	<14		>15
Piperacillin	Pseudomonas	100 µg	<17		>18
	Other Gram negatives	100 µg	<17	18-20	>21
Ticarcillin	Pseudomonas	75 µg	<14		>15
	Other Gram negatives	75 µg	<14	15-19	>20

Antimicrobial agent	When testing against	Disc content	Zone diameter nearest whole mm		
			Resistant	Intermediate	Susceptible
ß-LACTAM/ß-LACTAMASE INHIBITOR COMBINATIONS					
Amoxycillin/clavulanic acid	Staphylococci	20/10 µg	<19		>20
	Other organisms	20/10 µg	<13	14-17	>18
Ampicillin/sulbactam	Staph & Gram -ve	10/10 µg	<11	12-14	>15
Piperacillin/tazobactam	Pseudomonas	100/10 µg	<17		>18
	Other Gram negatives	100/10 µg	<17	18-20	>21
	Staphylococci	100/10 µg	<17		>18
Ticarcillin/clavulanic acid	Pseudomonas	75/10 µg	<14		>15
	Other Gram negatives	75/10 µg	<14	15-19	>20
	Staphylococci	75/10 µg	<22		>23
CEPHEMS					
Cefamandole		30 µg	<14	15-17	>18
Cefazolin		30 µg	<14	15-17	>18
Cefotaxime		30 µg	<14	15-22	>23
Cefoxitin		30 µg	<14	15-17	>18
Ceftazidime		30 µg	<14	15-17	>18
Ceftizoxime		30 µg	<14	15-19	>20
Ceftriaxone		30 µg	<13	14-20	>21
Cefuroxime oral		30 µg	<14	15-22	>23
Cefuroxime parentral		30 µg	<14	15-17	>18
Cephalothin		30 µg	<14	15-17	>18
CARBAPENEMS					
Imipenem		10 µg	<13	14-15	>16
MONOBACTAMS					
Aztreonam		30 µg	<15	16-21	>22
GLYCOPEPTIDES					
Telcoplanin		30 µg	<10	11-13	>14
Vancomycin	Enterococci	30 µg	<14	15-16	>17
	Other Gram positives	30 µg	<9	10-11	>12
AMINOGLYCOSIDES					
Amikacin		30 µg	<14	15-16	>17
Gentamicin	except high resistant enterococci	10 µg	<12	13-14	>15
Kanamycin		30 µg	<13	14-17	>18
Netilmycin		30 µg	<12	13-14	>15

Antimicrobial agent	When testing against	Disc content	Zone diameter nearest whole mm		
			Resistant	Intermediate	Susceptible
Streptomycin		10 μg	<11	12-14	>15
Tobramycin		10 μg	<12	13-14	>15
MACROLIDES					
Azithromycin		15 μg	<13	14-17	>18
Clarithromycin		15 μg	<13	14-17	>18
Erythromycin		15 μg	<13	14-22	>23
TETRACYCLINES					
Doxycycline		30 μg	<12	13-15	>16
Minocycline		30 μg	<14	15-18	>19
Tetracycline		30 μg	<14	15-18	>19
QUINOLONES					
Ciprofloxacin		5 μg	<15	16-20	>21
Enoxacin		10 μg	<14	15-17	>18
Lomefloxacin		10 μg	<18	19-21	>22
Nalidixic acid		30 μg	<13	14-18	>19
Norfloxacin		10 μg	<12	13-15	>16
Ofloxacin		5 μg	<12	13-15	>16
OTHERS					
Chloramphenicol		30 μg	<12	13-17	>18
Clindamycin		2 μg	<14	15-20	>21
Nitrofurantoin		300 μg	<14	15-16	>17
Rifampin		5 μg	<16	17-19	>20
Sulfonamides		250/300 μg	<12	13-16	>17
Trimethoprim		5 μg	<10	11-15	>16
Trimethoprim/ sulfamethoxazole		1.25/ 23.75 μg	<10	11-15	>16

* For *Vibrio cholerae*, the results of disc diffusion tests for ampicillin, tetracycline and trimethoprim/sulfamethoxazole correlate with results obtained by broth microdilution methods.

Results

The result of the susceptibility test, as reported to the clinician, is the classification of the microorganism in one of two or more categories of susceptibility. The simplest system comprises only two categories, susceptible and resistant. This classification, although offering many advantages for statistical and epidemiological purposes, is too inflexible for the clinician to use. Therefore, a three-category classification is often adopted. The Kirby-Bauer method and its modifications recognize three categories of susceptibility and it is important that both the clinicians and the laboratory workers understand the exact definitions and the clinical significance of these categories.

- **Susceptible:** An organism is called "susceptible" to a drug when the infection caused by it is likely to respond to treatment with this drug, at the recommended dosage.
- **Intermediate susceptibility** covers two situations. It is applicable to strains that are "moderately susceptible" to an antibiotic that can be used for treatment at a higher dosage because of its low toxicity or because the antibiotic is concentrated at the focus of infection (e.g. urine). The term also applies to those strains that are susceptible to a more toxic antibiotic that cannot be used at a higher dosage. In this situation the intermediate category serves as a buffer zone between susceptible and resistant.
- **Resistant:** This term implies that the organism is expected not to respond to a given drug, irrespective of the dosage and the location of the infection.

For testing the response of staphylococci to benzylpenicillin, only the categories 'susceptible' and 'resistant' (corresponding to the production of β-lactamase) are recognized. Staphylococci that are resistant to methicillin or oxacillin are also resistant to other penicillins or cephalosporins even though they show a zone of inhibition against these drugs.

Zone diameters, to the nearest whole mm for various antimicrobial agents with disc content specified for each one for interpretation as susceptible, intermediate and resistant are given in Table 2.

Quality assurance in susceptibility test

The precision and accuracy of the test are controlled by the parallel use of a set of control strains, with known susceptibility to antimicrobial agents. These quality control strains are tested using exactly the same procedure as for the test organisms. The zone sizes shown by the control organisms should fall within the range of diameters given in Table 3. When the results regularly fall outside this range, they should be regarded as evidence that a technical error has been introduced into the test, or that the reagents are at fault. Each reagent and each step in the test should then be investigated until the cause of the error has been found and eliminated.

The quality assurance programme should use standard reference strains of bacteria that are tested in parallel with the clinical culture. They should preferably be run every week, or with every fifth batch of tests, and in addition, every time that a new batch of Mueller-Hinton agar or a new batch of discs is used. The standard strains are:

Staphylococcus aureus (ATCC 25923)
Escherichia coli (ATCC 25922)
Pseudomonas aeruginosa (ATCC 27853)

Culture for day-to-day use should be grown on slants of nutrient agar (tryptic soya agar is convenient) and stored in a refrigerator. These should be subcultured onto fresh slants every two weeks.

Table 3: **Quality Control – Susceptibility of Control Strains**

Zone diameter of inhibition (mm)

Antibiotic	Disc potency (IU or μg)	*S.aureus* (ATCC 25923)	*E.coli* (ATCC25922)	*P.aeruginosa* (ATCC 27853)
Amikacin	30	20-26	19-26	18-26
Ampicillin	10	27-35	16-22	-
Ceftriaxone	30	22-28	29-35	17-23
Cephalothin	30	29-37	15-21	-
Chloramphenicol	30	19-26	21-27	-
Ciprofloxacin	5	22-30	30-40	25-33
Clindamycin	2	24-30	-	-
Erythromicin	15	22-30	-	-
Gentamicin	10	19-27	19-26	16-21
Nalidixic acid	30	-	22-28	-
Nitrofurantoin	300	18-22	20-25	-
Norfloxacin	10	17-28	28-35	-
Oxacillin	1	18-24	-	-
Penicillin G	10	26-37	-	-
Piperacillin	100	-	24-30	25-33
Tetracycline	30	19-28	18-25	-
Tobramycin	10	19-29	18-26	19-25
Trimethoprim	5	19-26	21-28	-
Trimethoprim-sulfamethoxazole	25	24-32	24-32	-

Biosafety

- Observe good laboratory practices as outlined in Chapter 8.
- Antimicrobial susceptibility of organisms such as *Yersinia pestis* and *Bacillus anthracis* should be performed under BSL-3 environment.
- The culture plates with organisms such as *Bacillus anthracis* be appropriately autoclaved after chemical treatment with hypochlorite solution before disposal.

Referral

- In the event of disease outbreak the susceptibility results must be verified by the reference laboratory.
- When unusual susceptibility patterns are encountered.
- As a part of quality assurance programme.

When intermediate laboratories need not undertake suscpetibility tests?

- In organisms with predictable susceptibility patters e.g. *Streptococcus haemolyticus, Corynebacterium diphtheriae* and *Treponema pallidum* infections.
- In cases where susceptibility tests are technically more demanding such as *Mycobacterium tuberculosis* and *Neisseria* spp.

Salient features of quality assurance in antibiotic susceptibility testing

- Use antibiotic discs of 6 mm diameter.
- Use correct content of antimicrobial agent per disc.
- Stock the supply of antimicrobial discs at -20^{0}C.
- Use Mueller-Hinton medium for antibiotic sensitivity determination.
- Use appropriate control cultures.
- Use standard methodology for the test.
- Use coded strains from time to time for internal quality control.
- Keep the antibiotic discs at room temperature for one hour before use.
- Incubate the sensitivity plates for 16-18 hours before reporting.
- Incubate the sensitivity plates at 35^{0}C.
- Space the antibiotic discs properly to avoid overlapping of inhibition zone.
- Use inoculum size that produces near confluent growth.
- Ensure an even contact of the antibiotic disc with the inoculated medium.
- Measure the zone sizes precisely.
- Interpret the zone sizes by referring to standard charts.

Further reading

NCCLS: Performance Standards for Antimicrobial Susceptibility Testing: Eighth Informational Supplement.M100-S8, Vol 18 No 1 January 1998, NCCLS, Pennysylvania, USA.

COMMUNITY HEALTH CELL
LIBRARY AND DOCUMENTATION UNIT
ST- 102

8. Safety in Laboratories

Laboratory safety is a vital component of functioning of any laboratory. Safety procedures and precautions to be followed in the microbiology laboratory should be designed to:

- ➢ Restrict microorganisms present in specimens or cultures to the vessels in which they are contained.
- ➢ Prevent environmental microorganisms (normally present on hand, hair, clothing, laboratory benches or in the air) from entering specimens or cultures and interfering with the results of the studies.

Laboratory biosafety levels

Four biosafety levels have been recommended based on the infectiousness of the agent/s.

Biosafety Level -1 (BSL-1): Adherance to standard microbiological practices. No special requirement as regards containment equipment.

Biosafety Level-2 (BSL-2): In addition to the use of standard microbiological practice, laboratory coats, decontamination of infectious wastes, limited access, protective gloves and display of biohazard sign and partial containment equipment are the requirements for this level.

Most peripheral and intermediate laboratories need BSL-1 or BSL-2 laboratory facilities.

BSL-3: In addition to BSL-2, it has special laboratory clothing, controlled access to laboratory and partial containment equipment.

BSL-4: BSL-3 plus entrance through change room where laboratory clothing is put on, shower on exit, all wastes are decontaminated before exit from the facility. It requires maximum containment equipment.

Laboratory facilities in BSL-2

- ➢ Laboratory should be designed in such a way that it can be easily cleaned.
- ➢ Laboratory contains a sink for washing.
- ➢ Laboratory tops are impervious to water but resistant to acids, alkalies and organic solvents.
- ➢ An autoclave to decontaminate infectious material is available.
- ➢ Illumination is adequate for all laboratory activities.
- ➢ Storage space is adequate.

Preventive measures against laboratory infections

These are aimed to protect workers, patients and cultures. Following steps are suggested:

- ➢ Perform adequate sterilization before washing or disposing waste.
- ➢ Provide receptacle for contaminated glassware.
- ➢ Provide safety hood.
- ➢ Ensure that tissues are handled and disposed of properly.
- ➢ Promote regular handwashing and cleaning of bench tops.
- ➢ Ensure use of gloves.
- ➢ Provide mechanical pipetting devices.
- ➢ Protect patients from laboratory personnel with skin or upper respiratory tract infections.
- ➢ Provide special disposal containers for needles and lancets.

Pipetting

Pipetting and suctioning have been identified as the significant and consistent causes of occupational infections. Various important precautions that must be taken while pipetting are:

- ➢ Develop pipetting techniques that reduce the potential for creating aerosols.
- ➢ Plug pipettes with cotton.
- ➢ Avoid rapid mixing of liquids by alternate suction and expulsion.
- ➢ Do not forcibly expel material from a pipette.
- ➢ Do not bubble air through liquids with a pipette.
- ➢ Prefer pipettes that do not require expulsion of last drop of liquid.
- ➢ Drop material having pathogenic organisms as close as possible to the fluid or agar level.
- ➢ Place contaminated pipettes in a container having suitable disinfectant for complete immersion.

A variety of pipettes are available. Selection should depend upon the ease of operation and the type of work to be performed.

Hypodermic syringes and needles

Accidents involving the use of syringes and needles while drawing blood from patients or performing experiments on laboratory animals are among the most common causes of occupational infections in laboratories and health care facilities. They account for almost

25% of the laboratory-acquired infections that occur by accidents. The practices which are recommended for hypodermic needle and syringes are:

- Avoid quick and unnecessary movements of the hand holding the syringe.
- Examine glass syringes for chips and cracks, and examine needles for barbs and plugs.
- Use needle locking (Luer Lock type) syringes only and be sure that needle is locked securely.
- Wear surgical or other gloves.
- Fill syringes carefully to minimize air bubbles and frothing.
- Expel excess air, liquid and bubbles vertically into a cotton pledget moistened with suitable disinfectant.
- Do not use syringe to forcefully expel infectious fluid into an open vial for mixing. Mixing with a syringe is appropriate only if the tip of the needle is held below the surface of the fluid in the tube.
- Do not bend, shear, recap or remove the needle from syringe by hand.
- Place used needle-syringe units directly into a puncture-resistant container and decontaminate before disassembly, reuse or disposal.

Opening containers

The opening of vials, flasks, petri dishes, culture tubes, embryonated eggs, and other containers of potentially infectious materials poses potential but subtle risks of creating droplets, aerosols or contamination of the skin or the immediate work area. The most common opening activity in most health care laboratories is the removal of stoppers from containers of clinical materials. It is imperative that specimens should be received and opened only by personnel who are knowledgable about occupational infection risks. Various precautions that can be taken in this regard are:

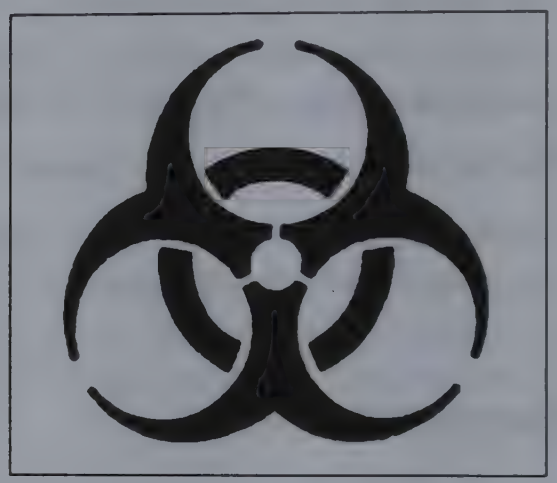
- Open containers with clinical specimens in well-lighted and designated areas only.
- Wear a laboratory coat and suitable gloves.
- If possible, use a plastic-backed absorbent paper towel to:
  - facilitate clean-up
  - reduce generation of aerosols
- Specimens which are leaking or broken may be opened only in safety cabinets.

Tubes containing bacterial cultures should be handled with care. Vigorous shaking of liquid cultures creates a heavy aerosol. When a sealed ampoule containing a lyophilized or liquid culture is opened, an aerosol may be created. Ampoules should be opened in a safety cabinet.

Laboratory access

- ➢ As far as possible children and pregnant women visitors should not enter the microbiological laboratories.
- ➢ Appropriate signs should be located at points of access to laboratory areas directing all visitors to a receptionist or receiving office for access procedures.
- ➢ The universal biohazard symbol (Fig 1) shall be displayed at specific laboratories in which manipulations of organisms with moderate and heavy risk are being carried out. Only authorized visitors shall enter the laboratory showing universal biohazard sign. Doors displaying biohazard symbol shall not be propped open, but shall remain closed when in use.

Figure 1: *Universal biohazard sign*



Clothing

- ➢ All employees and visitors in microbiological laboratories shall wear laboratory clothing and laboratory shoes or shoe covers.
- ➢ Disposable gloves shall be worn wherever radiological, chemical, carcinogenic materials or virus preparations of moderate to high risk are handled.
- ➢ Laboratory clothings including shoes shall not be worn outside the work area.

Accidents in laboratory

In the microbiological laboratory, bacterial infections pose the most frequent risk. The important diseases/organisms are:

Hepatitis B virus
HIV
Brucella spp.
Leptospires
Mycobacteria spp.
Histoplasma
Shigella spp.
Salmonella spp. including S typhi
Bacillus anthracis
Yersinia pestis

Accidents and spills

The order of priorities is as follows:

- ➢ Protection of personnel
- ➢ Confinement of contamination
- ➢ Decontamination of personnel
- ➢ Decontamination of area involved

Decontamination of skin. The area is washed thoroughly with soap and water. Detergents or abrasive materials must not be used and care must be taken not to damage the skin.

Decontamination of cuts\eyes. These are irrigated with water taking care to prevent the spread of contamination from one area to another.

Decontamination of clothing. Contaminated garments should be removed immediately and placed in a container. They should not be removed from the spill location until contamination has been monitored.

Decontamination of work surfaces

- ➢ Flood the total spillage area including the broken container with disinfectant.
- ➢ Leave undisturbed for 10 minutes.
- ➢ Mop with cotton wool or absorbent paper.
- ➢ Wear disposable gloves, apron and goggles.
- ➢ If a dustpan and brush or forceps have been used these too require disinfection.
- ➢ For blood or viruses, hypochlorites (10 gm/L) are used.
- ➢ Do not use hypochorite solution in centrifuges.
- ➢ Use activated gluteraldehyde (20 gm/L) on surfaces for viral decontamination.
- ➢ Place all potentially contaminated materials in a separate container and retain until monitored.
- ➢ Restrict the entry to such an area until contamination monitoring has been carried out.

Management of laboratory accidents

An adequately equipped first-aid box should be kept in the laboratory in a place that is known and accessible to all members of staff. The box must be clearly marked and preferably be made of metal or plastic to prevent from damage by pests. A medical officer should be consulted regarding the contents of the box. A first-aid chart giving the immediate treatment of cuts, burns, poisoning, shock and collapse, should be prepared and displayed in the laboratory.

General laboratory directions for safety

The salient general laboratory directions which must be obeyed by all are:

- ➢ Long hair should be bound back neatly away from shoulders.
- ➢ Do not wear any jewellery to laboratory sessions.
- ➢ Keep fingers, pencils, bacteriological loops etc. out of your mouth.
- ➢ Do not smoke in the laboratory.
- ➢ Do not lick labels with tongue (use tap water).
- ➢ Do not drink from laboratory glasswares.
- ➢ Do not wander about the laboratory; uncontrolled activities cause:
  - accidents
  - distract others
  - promote contamination
- ➢ Do not place contaminated pipettes on the bench top.
- ➢ Do not discard contaminated cultures, glasswares, pipettes, tubes or slides in wastepaper basket or garbage can.
- ➢ Avoid dispersal of infectious materials.
- ➢ Operate centrifuges, homogenizer and shakers safely.
- ➢ Immunize the laboratory workers against vaccine-preventable diseases such as hepatitis B, meningococcal meningitis, rabies, etc.

Further reading

1. Guidelines for Preventing HIV, HBV and other Infections in the Health Care Setting, SEARO WHO, Delhi 1996.
2. World Health Organization: Laboratory biosafety manual, 2nd Edition, WHO, 1993.
3. Miller B.M. (et al). Laboratory safety: principles and practices, Washington DC, American Society for Microbiology, 322, 1986.

9. Quality Assurance

Quality Assurance (QA) is a wide ranging concept covering all matters that individually or collectively influence the quality of a product. It denotes a system for continuously improving reliability, efficiency and utilization of products and services. In the context of quality assurance two important definitions need to be clearly understood:

(i) **Internal Quality Control (IQC):** which denotes a set of procedures undertaken by the staff of health facility (medical, paramedical workers as well as laboratorians) for **continuously** and **concurrently** assessing laboratory work so that quality results are produced by the laboratory for supporting quality health care at patient and community levels.

(ii) **External Quality Assessment (EQA):** is a system of objectively assessing the laboratory performance by an outside agency. This assessment is **retrospective** and **periodic** but is aimed at improving the IQC.

IQC and EQA are complementary in ensuring the reliability of the procedures, results and quality of the product.

What is the objective of QA?

QA programmes are required for the following reasons:

- To improve the quality of health care.
- To generate reliable, reproducible results.
- To establish inter-laboratory comparability in laboratory testing.
- To establish the credibility of the laboratory among doctors and the public at large.
- Motivating the staff for further improvement.
- Prevention of legal complications which may follow poor quality results.

Factors affecting the quality

It is commonly believed that the quality of laboratory results solely depends upon the laboratory undertaking this analysis. However, there are many pre-analytical and post-analytical factors which influence the quality of the end results to a very significant extent. The principle of "GIGO" – "Garbage in Gabage Out" very well applies to the laboratory tests also. Some of the important factors influencing quality are listed here:

(i) **Specimen:** This is the single most important factor. Selection of the right sample, collection in a right manner, adequate quantity, proper transportation to the laboratory, and processing of the sample before testing, are crucial factors.

(ii) **Personnel:** The quality of the laboratory results generated is directly proportional to the training, commitment and motivation of the technical staff.

(iii) **Environmental factors:** Inadequate lighting, workspace or ventilation or unsafeworking conditions may influence the laboratory results.

(iv) **Analytical factors:** The quality of reagents, chemicals, glassware, stains, culture media,use of standard procedures and reliable equipment all influence laboratory results. Failure to examine a sufficient number of microscope fields can lead to false negative results.

(v) **Post analytical factors:** Transcription errors, incomplete reports, and improper interpretation can adversely influence the laboratory results.

IQC – the mainstay of QA

The backbone of a good quality assurance programme is a good IQC. Intermediate and peripheral laboratories must put in place various IQC procedures and may participate in any EQAS that is in operation.

Requirements of IQC

- Comprehensive: Cover all steps from collection of sample to reporting.
- Regular continuous monitoring.
- Rational: Focus more on critical factors.
- Practical: Should not atttempt to evaluate everything.
- Economical: Should be cost-effective and within the provided budget.

Each laboratory should have Standard Operating Procedure Manuals (SOPMs) which should include the following information about the infrastructure of a laboratory

- Biosafety precautions
- Disposal of infectious waste
- Collection, transport and storage of specimens
- Criteria of rejection of samples
- Processing of specimens
- Maintenance of equipment
- Recording of results
- Reporting of results
- Procedure of quality control
- Referral

SOPMs should be periodically reviewed and revised and religiously followed in the laboratories.

Most of the above-mentioned factors have been described in various chapters of this document. The remaining have been presented briefly here.

Maintenance of equipment

Good quality equipment is absolutely essential to generate quality results. Care of the equipment purchased is also crucial. The quality control steps for some of the commonly-used equipment at the intermediate/peripheral laboratory level is depicted in Table 1.

Table 1: ***Suggested maintenance of commonly-used equipment***

Equipment	Maintenance Instructions
Autoclave	Clean and change water monthly Adjust water level before each run Record time, temperature and pressure for each run Inspect gasket in the lid weekly Technical inspection every six months
Incubator	Clean inside walls once in a month Record temperature at the start of each working day Technical maintenance every six months
Hot air oven	Clean the inside at least once a month Record time and temperature with every run Technical inspection every six months
Microscope	Wipe lenses with lens paper at the end of each day's work Protect the microscope from dust, vibrations and moisture Place a shallow plate containing dry blue silica gel in a box to absorb moisture Check alignment of the condenser once a month Technical inspection once in a year
Balance	Keep the balance and weights clean and dry Always use a container or weighing paper, do not put material directly on the pan Prevent the balance from drafts of air

Equipment	Maintenance Instructions
Refrigerator	Place at least 10 inches away from the wall Clean and defrost at least every two months Record temperature daily Technical service at least once a year
Water Bath	Check water level daily Check temperature before and during use Clean monthly Technical inspection once in six months
Inspissator	Check temperature daily Clean after each batch of culture media prepared
Centrifuge	Wipe inner walls with antiseptic solution weekly Check brushes and bearings every six months
Glassware	Discard chipped glassware Ensure these are free of detergents Do not store sterile glassware for more than three weeks before it is used.

Performance tests on culture media

Culture media may be prepared from the individual ingredients or may be prepared from dehydrated powders available commercially. The important points in QC of media are listed here:

- Do not over-stock the media. Store the required quantities only which can be used in 6-12 months.
- Store the media away from moisture by securing the caps of all the containers tightly.
- Store in a dark, cool and well-ventilated place.
- Keep a record of the receipt, and opening of the media container.
- Discard all dehydrated media that are either darkened or caked. Rotate the stock of media, following the principle of "first in, first out".
- For preparation of media adhere strictly to the manufacturer's instructions.
- Prepared media should be protected from sunlight and heat.
- Sterility testing, performance testing and pH test of the prepared media should be done as listed in Table-2.

Table 2: ***Performance tests on commonly-used media***

Medium	Incubation	Control Organism	Expected Result
Blood Agar	24h, CO_2	*S. aureus*	Growth and beta-haemolysis
		S.pneumoniae	Growth and alpha-haemolysis
Chocolate agar	24h, CO_2	*H.influenzae*	Growth
MacConkey agar With crystal violet	24h	*E.coli*	Red colonies
		P.mirabilis	Colourless colonies (no swarming)
		E.faecalis	No growth
Methyl red/Voges-Proskauer	48h	*E.coli*	Positive/negative
		K.pneumoniae	Negative/positive
Mueller-Hinton	24h	*E.coli* ATCC 25922	Acceptable zone sizes
		P.aeruginosa ATCC 27853	Acceptable zone sizes
Peptone water (indole)	24h	*E.coli*	Positive
		K.pneumoniae	Negative
Simmons citrate (incubate with loose screwcap)	48h	*E.coli*	No growth
		K.pneumoniae	Growth, blue colour
Thiosulfate citrate bile salt (TCBS) agar	24h	*Vibrio spp.* (non agglutinable	Yellow colonies
Thayer Martin Agar	24h, CO_2	*N.meningitidis*	Growth
		N.gonorrhoeae	Growth
		Staphylococci	No growth
		E.coli	No growth

Quality control for commonly-used tests

The QC for commonly-used tests at the intermediate/peripheral level is listed in Table 3.

Quality control of immunological tests

Quality control procedures used for the detection of antigen or antibodies by various test methods are listed in Table 4.

QA of antibiotic susceptibility testing

For details refer to chapter 7.

Table 3: ***QC for commonly-used tests***

Procedure/result	Test Control organism		Expected reaction
Catalase	*S. aureus*	+	Bubbling reaction
	Streptococcus spp.	–	No bubbling
Coagulase	*S. aureus*	+	Clot formation in 4 hours
Indole	*E. coli*	+	Red ring at surface
	E. aerogenes	–	Yellow ring at surface
Methyl red	*E. coli*	+	Instant red colour
	E.. aerogenes	–	No colour change
Oxidase	*P.aeruginosa*	+	Purple colour in 20 seconds
	E. coli	–	No colour in 20 seconds
Voges Proskauer	*E. aerogenes*	+	Red colour
	E. coli	–	No colour change
Bacitracin disc	*Streptococcus group A*	+	Zone of inhibition
	E. faecalis	–	Zone of inhibition
Optochin disc	*S. pneumoniae*	+	Zone of inhibition
	S. viridans	–	No zone of inhibition
Oxidase disc	*P.aeruginosa*	+	Purple colour in 30 seconds
	E. coli	–	No change in colour

The testing should be done each time a new batch of working solution is prepared.

Table 4: ***QC procedures for immunological tests***

Test	Control procedures required	Expected results
1. Flocculation test (RPR)	Nonreactive serum control	No clumping
	Weakly reactive serum control	Clumping of graded activity
	Reactive serum control	Clumping of graded activity
2. Latex agglutination test (ASO)	Negative control serum	No clumping
	Positive control serum	Clumping
3. Direct agglutination (Widal test)	Antigen control	No clumping
	Negative control serum	No clumping
	Positive control serum	Clumping
4. Capsular Quellung reaction (Omni serum, *H.influenzae* type b)	Pneumococci	Capsular swelling
	Haemolytic streptococci	No reaction
	H.influenzae type b	Capsular swelling
	Acinetobacter anitratum	No reaction

In service training of staff

Periodic updating of the skills and knowledge of the laboratory workers is essential for maintaining quality. Course-curriculum of such trainings should focus on the issues highlighted above.

Participation in external quality assessment

Participation in EQAS reassures about the correctness of the results generated by the laboratory and finds out whether IQC is in place or not. The control or referral laboratories should organise EQAS in some commonly used tests.

Further reading

1. Sudarshan Kumari, Rajesh Bhatia, CC Heuck: Quality Assurance in Bacteriology & Immunology WHO Regional Publication, South-East Asia Series No. 28, 1998.
2. Bhatia Rajesh & Ichhpujani R.L.: Quality Assurance in Microbiology CBS Publishers and Distributors, New Delhi, 1995.
3. Howaritz PJ, Kowanitz JH: Laboratory quality assurance (McGraw Hill Book Companry, New York (1987).

Section B
Guidelines for Standard Procedures for Specific Diseases

10. Cholera

Cholera is caused by various toxigenic species of the genus *Vibrio* especially *Vibrio cholerae* O1 and O139 as well as *V.parahaemolyticus*. The clinical manifestations of cholera also resemble acute gastroenteritis which is caused by a variety of other bacterial and viral agents.

In most cases, cholera is an acute diarrhoeal disease characterized by loss of large amount of fluids and electrolytes which, if untreated, may lead to cardiovascular collapse and death. For the most part, significant propagating epidemics of cholera are limited to areas with poor sanitation, but the possibility of appearance of imported cases in areas with good sanitation also exists.

Causative organism

Vibrio cholerae, the causative organism of cholera is a Gram negative, slightly curved, motile, rod-shaped bacterium readily isolated from the stool of the patients with this disease. It is oxidase and catalase positive

Specimens

(1) Faeces (preferred specimen)

(2) Rectal swab

Collection, storage and transportation of specimens

Stool specimens should be collected early, preferably within the first 24 hours of illness, and before the administration of any antimicrobial agents. Fluid stool, often "rice-water" in appearance, may be collected by inserting a petrolatum-lubricated soft rubber catheter into the rectum. Rectal swabs may also be employed, although highly efficient in the acute phase of illness, they probably are less satisfactory for culturing convalescent cases or suspected carriers.

Transport: Whenever possible, stool or rectal swab specimens should be inoculated on isolation plates with minimal delay. Viability of *V.cholerae* is well maintained in the alkaline milieu of "rice-water stool" but is unpredictable in formed stools. *V. cholerae* is very susceptible to desiccation hence, specimens must not be allowed to dry.

When specimens cannot be cultured without delay, especially when they must be transported by courier, rectal swabs or stool material should be placed in the modified semisolid transport medium of Cary and Blair. The efficiency of this medium in maintaining viability of *V.cholerae* for upto 4 weeks has been verified. In the absence of available suitable transport media, strips of blotting paper may be soaked in rice-water stool and inserted into air-tight plastic bags. Specimens collected in this way may remain viable for up to 5 weeks.

Storage: Specimens in transport media may be shipped to the laboratory without refrigeration. Refrigeration, but not freezing is preferable for delayed transportation of raw collected stool specimens.

Salient features of specimen collection

- Faeces specimen should not be contaminated with urine.
- Do not collect specimen from bed pan.
- Collect the specimen in the early phase of the disease and, as far as possible, before the administration of antimicrobial agents.
- 1 to 2 gm quantity of faeces is sufficient.
- If possible, submit more than one specimen on different days.
- The fresh stool specimen must be received within 1-2 hours of passage.
- Store at 2-8°C, if not processed immediately.
- Modified Cary and Blair medium (see chapter 5) is recommended as a good transport medium. It is a very stable medium and can be stored for use in screw capped containers. It is a semi-solid transport medium. At least two swabs should be inoculated.
- Alternative transport media are Venkataraman-Ramakrishnan medium (V-R fluid) or alkaline peptone water.VR fluid should be prepared in 30 ml (1 oz) screw capped bottles (MacCartney bottles). It preserves vibrios for more than 6 weeks and has also proved to be very convenient medium for transportation as it can be kept at room temperature after collection of specimen.
- If none of the transport medium is available, collect watery stool from suspected cholera case by soaking blotting paper strip in the stool, sealing the strip in a polythene bag or an airtight container where the strip does not dry. The moist blotting paper strip can then be transported to the laboratory. Blotting papers have been found to be superior to the filter paper as the former can soak more moisture and retain the same over longer periods thus maintaining the viability of vibrios absorbed on it.

Materials

(a) Media

Transport medium:	Modified Cary and Blair
Primary inoculation:	MacConkey agar
	TCBS
	BSA
Enrichment medium:	Alkaline peptone water

(b) Reagents Oxidase reagent
V.cholerae O1, O139 antisera

Procedure

Direct Demonstration of Organism

Direct demonstration of *V.cholerae* is not recommended for general purposes. However, darting motility and its inhibition of specific antisera, especially after brief inoculation in broth, has proven valuable in experienced hands for presumptive diagnosis. The method is as follows:

- Prepare a hanging drop from alkaline peptone water which has been inoculated with faeces and incubated at 37°C for 4-6 hours.
- Examine it under high power dry lens of microscope.
- Darting motility characteristic of vibrios is suggestive of diagnosis.
- If available, add one drop of *V.cholerae* O1 antiserum (without preservative) to hanging drop preparation and reexamine for motility. Immobilization of organisms shall give a presumptive diagnosis of cholera.

Primary inoculation

It can be directly done on thiosulphate citrate bile salt sucrose agar (TCBS) or bile salt agar (BSA) media with or without enrichment in alkaline peptone water (APW).

- Inoculate a loopful of stool suspension in 5-10 ml alkaline peptone water (pH 8.2) and incubate at 37°C for 4-6 hours.
- Streak a loopful of the culture taken from surface of APW on the plating medium.
- Transfer a small aliquot into a fresh tube containing APW and incubate overnight at 37°C. Again inoculate on TCBS/BSA plates.
- Incubate at 35-37°C.

Examination of culture

(a) TCBS plate:

- Examine the TCBS plate for colony colour and growth characteristics associated with *Vibrio spp.* Colonies of *V.cholerae* are yellow in colour and profuse on TCBS. *V.parahaemolyticus* also gives good growth but the colonies are blue-green in colour and mucoid in consistency.
- Hold TCBS agar plate for 48 hours before reporting as negative.
- Some coliforms, *Proteus spp.* and *Enterococcus spp.* may grow, but colonies are usually small and translucent. Sucrose-fermenting *Proteus spp.* produce yellow colonies and must be carefully differentiated from *Vibrio spp.*. *Pseudomonas* and *Aeromonas* also grow on TCBS, but they usually form blue colonies.

(b) Other media

- On BSA, vibrios grow as small, transluscent and raised colonies.
- *Vibrio spp*. are capable of growing on routine laboratory media since the salt content of these media is sufficient to support the growth of halophilic organisms. On MacConkey agar medium *Vibrio spp*. grow as lactose nonfermenting colonies. In such instances, one can proceed as per chart 1.

Biochemical identification

Perform biochemical identification on suspect colonies on TCBS or BSA. Vibrios are oxidase positive and ferment glucose and sucrose with the production of acid only.

Oxidase test

1% Kovac's reagent (tetramethyl-p-phenylene diamine dihydrochloride) is required for the performance of this test. The test can be performed by either of the following two methods:

(a) Direct plate method:

- Pour 2-3 drops of Kovac's reagent directly on the isolated bacterial colonies on bacteriological medium plate.
- Observe the change in colour of the colonies.
- Vibrio colonies turn deep blue within 10 seconds.

(b) Indirect paper strip method:

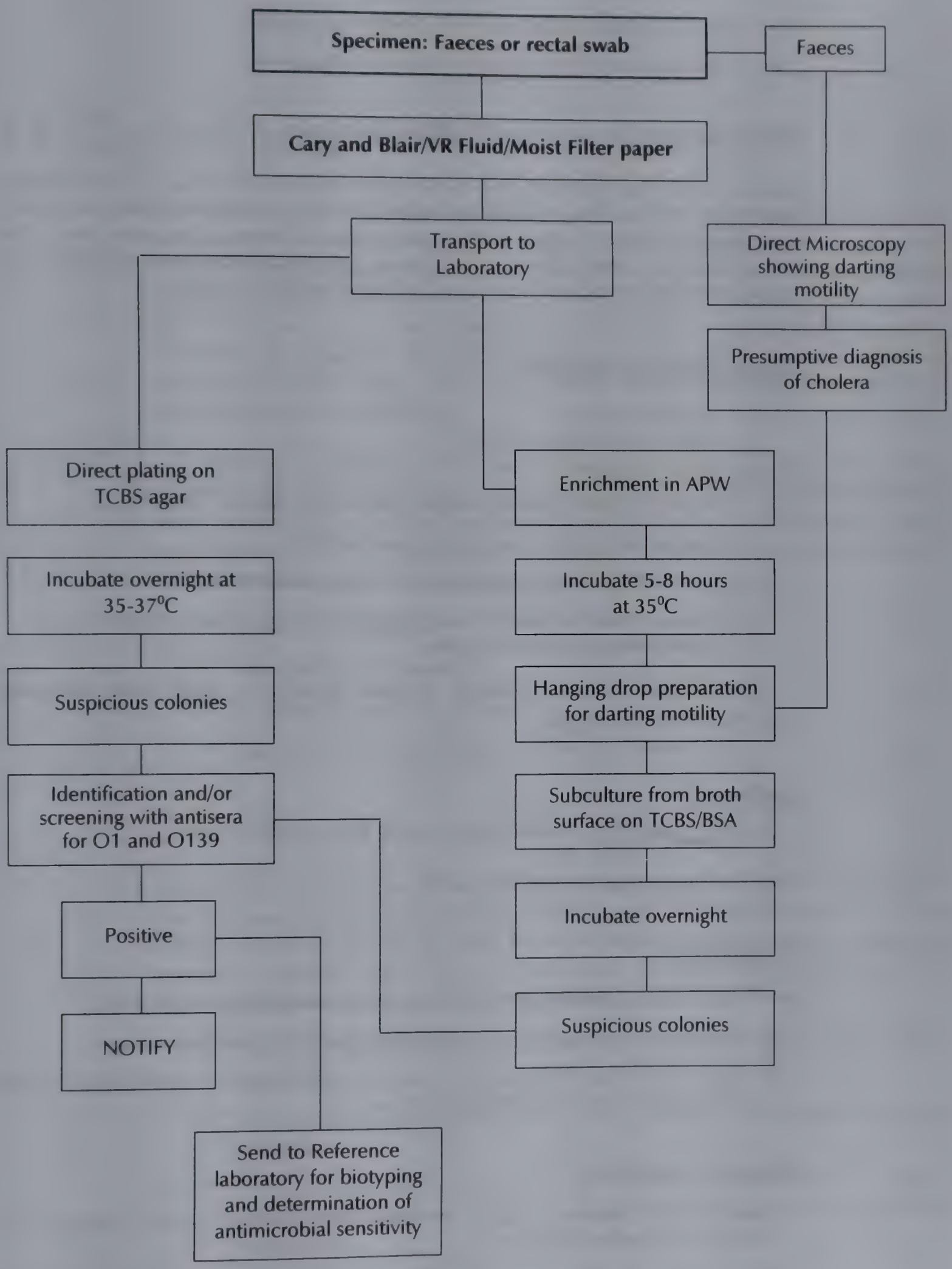
- Use filter paper strips to which Kovac's reagent has been added or procure commercially available discs or reagent impregnated strips.
- With the help of a platinum loop (or glass rod) pick up a loopful of suspected colonies and smear these on the strip. Do not use nichrome wire loop since it may yield a false positive result.
- Oxidase producing organisms turn the strip deep blue at the site of smear.
- Use *Pseudomonas aeruginosa* or *Vibrio cholerae* as positive control and *Escherichia coli* as negative control for quality control purposes.

Serological identification of *V.cholerae*

Test organism is identified as *V.cholerae* against *V.cholerae* O1 and O139 antiserum. This can be done from culture plates with or without biochemical test facilities.

- Subculture suspicious colonies from BSA heavily on nonselective medium.
- After 18 hours of incubation at 35-37°C, prepare a milky organism suspension.
- On a glass slide, mix one drop of organism suspension and one drop of antiserum (O1). If negative, repeat with O139 antiserum.
- Rotate slide for one minute and observe for agglutination.
- Positive agglutination confirms the identity of the organism and serotype.
- Similarly, with the help of Ogawa and Inaba antisera, the subtype can be determined. Isolates which agglutinate with both Ogawa and Inaba antisera belong to Hikojima serotype.

Chart 1: *Flow Chart for Diagnosis of Cholera*


Specimen: Faeces or rectal swab
Faeces
Cary and Blair/VR Fluid/Moist Filter paper
Transport to Laboratory
Direct Microscopy showing darting motility
Presumptive diagnosis of cholera
Direct plating on TCBS agar
Enrichment in APW
Incubate overnight at 35-37°C
Incubate 5-8 hours at 35°C
Suspicious colonies
Hanging drop preparation for darting motility
Identification and/or screening with antisera for O1 and O139
Subculture from broth surface on TCBS/BSA
Positive
Incubate overnight
NOTIFY
Suspicious colonies
Send to Reference laboratory for biotyping and determination of antimicrobial sensitivity

Quality assurance

- Follow manufacturer's instructions for media and antisera.
- Follow standard QA procedures for laboratory equipment.

Antimicrobial susceptibility testing

Resistant vibrios are extremely rare and, since antimicrobial therapy is not essential, susceptibility tests are of largely academic interest. Therapy of cholera is primarily dependent on adequate intravenous and/or oral fluid and electrolyte therapy. Oral tetracycline is the drug of choice as a useful adjunct to fluid therapy, resulting in more rapid elimination of *V. cholerae* from the gastrointestinal tract and a shortened period of diarrhea.

Reporting of results

(a) Negative cultures
Report no *V.cholerae* isolated

(b) Positive culture
Report *V.cholerae* isolated and also indicate the serotype , if confirmed.

(c) Biotype and serotype can be communicated later.

(d) If extended incubation is continued the report should indicate this.

(e) Mention about the drug of choice for treatment and non-requirement of antimicrobial susceptibility testing.

Since cholera is an internationally notifiable disease, the public health authorities must be notified accordingly.

Referral

- For further typing
- For antimicrobial susceptibility testing

Biosafety

- Wash hands particularly after collection and handling of clinical specimen.
- Use leak-proof containers in a sealed plastic bag for transport and storage.
- Disposal of unused faeces by incineration, autoclaving or disinfecting with 1% calcium or sodium hypochlorite for 6 hours.

Further reading

1. Barua D. 1974. Laboratory Diagnosis of Cholera P.85-126 In D.Barua and W.Burrows (ed.) Cholera. WB Saunders, Philadelphia.

2. Wachsmith Kaye I et al. Isolation and identification of *Vibrio cholerae* 01 from faecal specimens in *Vibrio cholerae* and cholera – molecular to global perspectives. ASM Press, Washington DC, 1994.

11. Diphtheria

Diphtheria is an acute infectious disease primarily of upper respiratory tract but occasionally of skin also. It is caused by toxigenic strains of *Corynebacterium diphtheriae*. There are three biotypes of this organism – gravis, intermedius and mitis. Recently a fourth biotype – belfanti has also been described. Characteristically *C.diphtheriae* causes a typical form of infection which is manifested by a greyish – white membrane at the site of the localization of organisms. This organism produces a powerful exotoxin capable of damaging the heart, kidney and muscles. Some toxigenic strains of *Corynebacterium ulcerans* can also cause diphtheria.

Diphtheria is a serious disease and diagnosis used to be essentially clinical. With changing epidemiology of disease it is becoming difficult to diagnose diphtheria purely on clinical grounds. As the disease is uncommon, many clinicians may not have seen a case of diphtheria and may miss clinical diagnosis. Laboratory is required to confirm the diagnosis. The laboratory diagnosis is described under following heads:

- Collection of specimen
- Demonstration of organism
- Isolation of organism by culture
- Toxigenicity test (usually done in reference laboratories)

Collection and transportation of specimen

In suspected cases, whether of fauceal or nasal diphtheria, swabs should be taken both from the throat and the nose. Preferably two swabs should be collected from the site most affected. Swabs should also be taken from skin lesions and wounds where diphtheria infection is suspected. Ideally the specimen should be collected by a doctor, nurse or experienced laboratory personnel. Following directions should be followed:

- Ensure the availability of sterile cotton swabs in test tube, tongue depressor and source of ligh.
- Ask the patient to open his mouth without putting out his tongue and to say Ahhhhh.....
- While the patient is saying Ahhhhh, press the outer two third of the tongue down with the tongue depressor, using the left hand, making the tonsils and back of the throat visible.
- Introduce the swab with right hand, avoiding touching the tongue.

- Locate the inflammed part of the throat which is red or white and located on the tonsils or fauces.
- Rub the swab firmly against the inflammed part, turning it round, and collect membrane, if present.
- If nothing abnormal is seen, swab the tonsils, the fauces and the back of the soft palate.
- Take two swabs and immediately plug the same in sterile test tubes.
- Similarly collect swabs in duplicate from the nose or skin lesions, if present.
- If swabs are to be transported long distance, these should be inoculated on Loeffler's serum slopes and retained within the container which is screw capped after breaking the part of the wooden stick of swab that remains out of container.

Storage

Specimens should be transported and processed as soon as possible. Delays of over 48 hours are undesirable. If processing is delayed, refrigeration is preferable to storage at ambient temperature.

Processing of swabs

- Use one swab for inoculating sheep blood agar, Loeffler serum slope and blood tellurite agar.
- If culture facilities are not available, streak this swab on Loeffler serum slope which may be used as a transport medium.
- With the second swab, streak two slides using a rolling movement to make sufficiently thick smears.
- If there is only one swab it is mandatory to first inoculate the culture media and then prepare the slides to avoid contamination as well as to ensure adequate material for culture.

Staining of smears

- In all cases, stain one of the smears with Gram stain (Chapter 4).
- If Gram stained smear shows morphology (described below) suggestive of *C.diphtheriae*, proceed to do Albert staining (Chapter 4) which demonstrates the presence or absence of metachromatic granules.

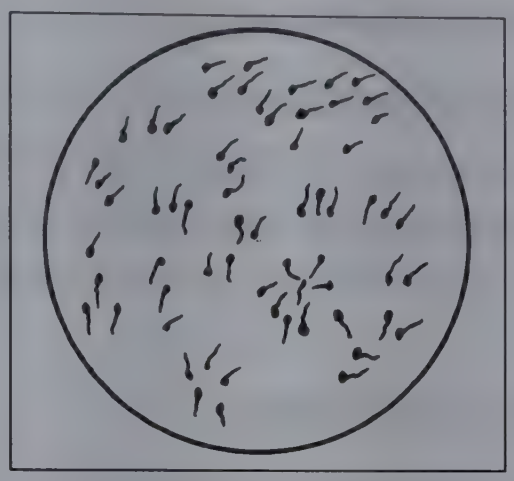
Morphology of *C.diphtheriae*

These are thin Gram positive bacilli, straight or slightly curved and often enlarged (clubbing) at one or both ends and are arranged at acute angles giving shapes of Chinese letters or V shape which is characteristic of these organisms (Fig 1). Present in the body of

the bacillus are numerous metachromatic granules which give the bacillus beaded or barred appearance. These granules are best demonstrated by Albert's stain.

These organisms have to be differentiated from commensal bacteria in throat called diphtheroids which are thick and short in appearance and do not contain metachromatic granules. These also do not exhibit Chinese letter arrangement. These exhibit a strong Gram positive reaction as compared to *C.diphtheriae* organisms which are weakly Gram positive.

Figure 1: *Morphology of C.diphtheriae*



Culture

Wherever facilities exist, attempt should be made to isolate the organism. Three media are used routinely for this purpose:

- **Sheep blood agar**: It supports the growth of *C.diphtheriae* as well as all other common bacterial pathogens.
- **Loeffler serum slope**: It is not a selective medium but gives abundant growth and morphology of *C.diphtheriae* is best developed. This medium can also be used as a transport medium.
- **Blood tellurite agar**: This is a selective medium which enhances the chances of isolation of *C.diphtheriae* from clinical specimen in which commensal bacterial flora (as in throat swab) may also be present.

Procedure

- Using a freshly collected swab from the patient, streak a blood agar plate, blood tellurite agar plate and inoculate in the water of condensation of Loeffler serum slope.
- Incubate aerobically at 35°C.
- After 4-6 hours of incubation, prepare smear from Loeffler serum slope and stain with Gram stain and Albert stain.
- If any organism suggestive of *C.diphtheriae* is visible, further subculture on blood tellurite agar.

- Examine a blood tellurite agar plate at intervals of 24 hours and 48 hours incubation and any growth (black in colour) should be again stained with Gram and Albert stain.
- If the culture shows organisms resembling diphtheria bacilli, despatch the growth on Loeffler serum slope to the nearest reference laboratory for confirmation, biotyping and toxigenicity testing.

Reporting of results

It could be one of the followings:

- Negative for *C.diphtheriae*.
- Morphologically suggestive of *C.diphtheriae*.
- Morphologically and culturally suggestive of *C.diphtheriae*. Isolate sent to reference laboratory for confirmation and toxigenicity determination.

Quality assurance

Quality control checks on stains and media to be performed with known positive and negative organisms. Use *Staphylococcus epidermidis* as the negative control and known *C.diphtheriae* as the positive control

Other significant organisms isolated from throat swabs

Throat swabs contain a variety of micro organisms including saprophytic *Neisseria* sps. *Neisseria meningitidis* which is a Gram negative diplococcus is an important organism which could cause disease or be present in a carrier state. *Haemophilus influenzae* is another important organism present in throat. Beta haemolytic streptococci, *Staphylococcus aureus* and *Candida spp*. are also frequently encountered

Important points about diagnosis of diphtheria

- Diagnosis of diphtheria is a laboratory emergency and deserves utmost priority.
- Mild cases of diphtheria resemble pharyngitis and pseudomembrane may be lacking.
- Diphtheria is no longer diagnosed easily on clinical grounds.
- Even non-toxigenic strains of *C.diphtheriae* are important. These have the potential to cause invasive disease in some patients and also the potential to undergo lysogenic conversion to toxin producers *in vivo* leading to disease.
- Specimen should be transported and processed as soon as possible.

Susceptibility to antimicrobial agents

Since *Corynebacterium diphtheriae* has a predictable antibiotic sensitivity pattern and penicillin (erythromycin in those who are sensitive to penicillin) continues to be the drug of choice for treatment, intermediate level laboratories should not attempt testing for susceptibility. Instead the isolate should be sent to the designated referral laboratory for this purpose, who can periodically give feed back to all the levels about the antibiotic sensitivity patterns of *C.diphtheriae*.

Biosafety

(a) All work on suspected *C.diphtheriae* isolates which is likely to generate aerosols must be performed in a safety cabinet.

(b) All other laboratory biosafety procedures as outlined earlier should be adhered to.

Referral

The isolate of *Corynebacterium diphtheriae* may be referred to the reference laboratory for

- ➢ Biotyping and toxigenicity testing
- ➢ Antimicrobial susceptibility testing
- ➢ Isolates associated with outbreaks and where epidemiologically indicated

Further reading

1. Brooks R and Joynson DHM, Bacteriological diagnosis of diphtheria. J.Clin. Pathol. 43, 576-80, 1990.
2. Facklam RR, Lawrence DN, Sottnek FO, Modified Culture techniques for *Corynebacterium diphtheria* isolation for desiccated scrabs. J.Clin. Microbio. 7, 1378, 1978.
3. Kisley SR, Price S and Ward T. *Corynebacterium ulcerans* – a potential cause of diphtheria. Commun. Disease Rev. 4:No.5, R63, 1994.

12. Enteric Fever

Typhoid fever is caused by *Salmonella typhi*. Paratyphoid fever is caused by *Salmonella paratyphi* A, B and C. The term enteric fever encompasses both typhoid and paratyphoid fevers. *S.typhi* is Gram negative, non acid fast, non capsulated and non-sporing bacilli, which measure approximately 2-4 µm x 0.6 µm. The organism is motile. Typhoid bacilli grow rapidly on ordinary media and optimum temperature of growth is 37°C.

The laboratory diagnosis of enteric fever depends upon:

- Isolation of the organism.
- Titration of circulating antibody against causative agent and demonstration of a four-fold or more rise in titre in two samples of serum that have been collected at least two weeks apart.

Collection of specimens

Blood (or bone marrow or clot), stool and urine are the clinical samples from which the causative agent can be isolated. However, the isolation rate is different at different stages. The best isolation rates from blood have been seen in the first week of illness, with faeces in the second week and with urine the third week of illness. Two serum samples are collected at an interval of at least 10-14 days to demonstrate four-fold change in antibody titre.

For general instructions on collection of specimens, refer to Chapter 2.

Isolation of the organism

Blood culture

- The organisms can be best isolated during the first 7-10 days of the illness, but in at least half the cases the organisms can still be isolated in the second and the third weeks.
- Add a minimum of 10 ml of venous blood from an adult patient to each of 50 ml of sodium taurocholate broth and glucose broth. From children at least 5 ml blood should be inoculated in each of the bottles.
- Incubate the inoculated media at 37°C overnight and subculture onto MacConkey agar and blood agar.

- Extend the incubation of blood culture bottle upto 7 days if subcultures fail to yield any bacterial growth. Subculture again on day 7 and if no growth is obtained blood culture may be declared as negative for enteric fever bacilli.
- Periodic subcultures are made after day 2, 5 and 7 on MacConkey agar. No growth after seven days may be regarded as negative. Very rarely organisms may appear as colonies after 30 days of incubation. However, it may not be feasible to maintain cultures that long.

Clot culture

The clot culture has following advantages over blood culture namely

- Serum is available for antibody titration.
- Special culture bottles are not required; so can be useful under field conditions.

The clot can be put in blood culture bottles and can be broken either mechanically or by adding streptokinase, if available.

Culture of faeces

- In at least 50% of the cases culture of the faeces is positive in first week, so faecal culture should be attempted in the first week of illness itself.
- The isolation of *S.typhi* greatly increases in second or third week.
- Rectal swabs give inferior results than faecal culture.
- Culture on desoxycholate citrate agar (DCA) and Wilson and Blair bismuth sulphite brilliant green agar using selenite F as enrichment medium. XLD agar gives better results in isolating salmonellae from faeces. It is recommended to use two enrichment media and two selective media to improve the isolation rate.

Urine culture

Urine culture is positive in a quarter to one third of cases, but there seems to be no regular excretion pattern in the urine. Bacteriuria is usually limited to the first few weeks of the illness and in great majority of the patients the urine is free from organisms weeks before the faeces becomes negative.

Cultural characters

The growth on MacConkey agar/DCA is pale yellow (non lactose fermenting) colonies, 2-3 mm in diameter, moist, circular and smooth convex surface. These are catalase positive and oxidase negative. The biochemical reactions which differentiate *S.typhi* from other genera are:

Glucose fermentation	+
Mannitol fermentation	+
Lactose fermentation	–
Sucrose fermentation	–
H_2S production	–
Phenylalanine deaminase	–
Citrate utilization	+

Slide agglutination

If on the basis of biochemical reactions, the organism has been identified as *Salmonella*, its identity can be confirmed with slide agglutination test, using polyvalent 'O' and polyvalent 'H' antisera against salmonellae. For identification monovalent 'O' specific antiserum for *S.typhi* is factor 9. To perform this test 1 drop each of normal saline is placed at two different sites on a clean glass slide. A loopful of biochemically suspect colony of *Salmonella* is emulsified in both. One of these is kept as central and another is tested first with polyvalent 'O' antiserum against Salmonella. If this gives visible agglutination within 2 minutes, the process is repeated with polyvalent 'H' antiserum and then with factor 9 antiserum.

A flowchart for examination of blood from a case with PUO as well as another regarding examination of faeces are appended as Chart 1 & Chart 2 to facilitate processing in the laboratory.

Antimicrobial susceptibility testing

The emergence and spread of *S.typhi* resistant to multiple antibiotics has assumed a massive proportion. Multi-drug resistant typhoid fever has become endemic in many developing countries. For this reason conventional antibiotics such as chloramphenicol, ampicillin and trimethoprim sulfamethoxazole can no longer be considered as the first line drugs in the treatment of typhoid fever. Each and every isolate of *S.typhi* should be subjected to antimicrobial susceptibility test using the method as detailed in Chapter 7. This will not only help in the management of the patient more efficiently but will also help in generating surveillance data which will be useful to formulate an antibiotic policy in a given hospital.

Serodiagnosis

Detection of antibodies

A large number of serological tests have been devised to detect and titrate the antibodies against common agents of enteric fever. The tube agglutination test (Widal test) has great historical but little diagnostic importance unless four fold rise is exhibited in two samples drawn at interval of 10-14 days from the start of the illness.

Widal test

In this test the patient's serum is tested by tube agglutination test for its titres of antibodies against H,O and Vi suspensions of enteric fever bacteria that are commonly encountered in an area such as *S.typhi* and *S.paratyphi* A.

Procedure for widal test

- Make a series of serum dilutions for each antigen to be tested. Include tubes with 0.5 ml saline for control of each antigen to be used.
- Use perfectly clean and dry test tubes and prepare dilutions beginning with 1:10 and doubling through 1:320 or so.

- Add 0.1 ml of serum to 0.9 ml of physiological saline and then dilute serially by mixing 0.5 ml diluted serum with 0.5 ml saline and discarding 0.5 ml from the last tube.
- From specimen submitted to detect possible rise in titre, prepare a series of 10 dilutions, ending with 1:5120.

Preparation of antigen

Use the killed standardized suspension of *S.typhi* O, *S.typhi* H, *S.paratyphi* AH and *S.paratyphi* AO. These are commercially available and their preparation should be undertaken as per the instructions of the manufacturers.

Procedure of test

- Shake the antigen suspension to ensure even distribution.
- Add 0.5 ml to each serum dilution and to saline for controls.
- Incubate the test overnight at 37°C and let tubes stand at room temperature for 15-20 minutes before reading.

Reading

In all cases, look first at the control tubes and proceed only if they show satisfactory results. There should be no appreciable sedimentation of the bacteria. Although a fine button may have been deposited at the bottom of the tube, no discernible clearing should have occurred. The antigen suspension should be evenly distributed as a rule.

Pick up the individual tubes of each row of the patient's specimens and look first at the supernatant. When a complete agglutination occurs, practically all the bacteria are removed from the supernatant which appears absolutely clear. When the reaction is negative the suspension should look as turbid as the antigen control is. The in-between reactions can be categorized into +, ++, +++.

Report

Give name of the test and antigen used, with titres, i.e. reciprocal of the highest dilution in which the reading is at least ++ for the specified antigen.

Inspite of overwhelming scientific evidence to the contrary, Widal test is still considered, as diagnostic of active infection if a four fold or more rise in antibody titres can be demonstrated between the two samples that have been collected at a gap of 10-14 days or say during the first week of illness and third week of illness or it can also indicate disease if a four fold or more fall can be demonstrated in two samples collected during acute illness and convalescence stages. Various studies have shown limited utility of Widal test in diagnosis of typhoid fever. While interpreting the test following factors must be considered:

- Repeated subclinical or past clinical infection in areas endemic for typhoid fever result in repeated boosting-up of immune system and the otherwise healthy individuals in the community may have high and variable antibody titres against *S.typhi*.

- 'O'antigens of salmonellae cross-react with certain other bacteria especially members of the family enterobacteriaceae. Infection with any of these organisms shall result in formation of antibodies that shall react with antigen suspension prepared from causative agents of enteric fever.
- Immunization with typhoid vaccines shall give rise to antibodies which may persist for a long time.
- The early treatment of cases of enteric fever with antibiotics may have a profound effect on antibody response. If antibodies have not appeared when treatment is begun, they are unlikely to do so subsequently and if these are already present no further rise in titres may occur.
- The Widal test can give significant reading in healthy carriers.
- 5-10% of the patients do not respond with antibody formation even after documented *S.typhi* infection.

The situations which give rise to false positive and false negative Widal test are :

False positives	Immunization with typhoid vaccine
	Repeated subclinical infection
	Past clinical infection
	Healthy carriers of *S.typhi*
	Anamnestic response
	Patients of cirrhosis and hepatitis
False negatives	Too early collection of blood sample
	Patients on antibiotics
	5-10% patients do not respond by antibody formation
	Immunosuppressed patients

In view of the various factors influencing the results of Widal test giving rise to false positive and false negative results, it is better not to rely too much on the Widal test. Every attempt must be made to culture the organisms from clinical specimens.

Reporting of results

(a) If culture positive, report as POSITIVE result, specifying the species. Provide the antibiotic sensitivity pattern to the clinician.

(b) If negative, report as NEGATIVE, mentioning the days of incubation.

(c) If Widal test done on paired sera shows 4-fold rise of antibody titre report as suggestive of typhoid fever.

(d) In single serum sample or absence of 4-fold rise of titres – No comments possible.

Quality assurance

(1) Practice all the internal quality control measures as detailed in chapter 9.

(2) Preferably participate in an external quality assessment programme on identification of *Salmonella*, Widal test and antimicrobial susceptibility testing.

Diagnosis of chronic typhoid carriers

In order to label a person as a typhoid carrier, the isolation of the organism is a must. The isolation can be made from urine and faeces. If these are repeatedly negative, bile or duodenal aspirate can be used. Vi antibodies present in a titre of more than 1:10 is also suggestive of chronic typhoid carrier.

The special points to be remembered in the diagnosis are:

- ➢ Repeated cultures are usually required.
- ➢ Enrichment media should be used.
- ➢ Variety of culture media such as MacConkey, DCA etc. may be tried.

Biosafety

(1) Observing the general biosafety measures as outlined in Chapter 8 is sufficient.

(2) If feasible, workers in the laboratory can be protected by using oral vaccine (Ty21a) or injectable Vi polysaccharide vaccine.

Referral

(1) For serotyping and phage typing.

(2) Isolates from outbreaks or from similiar epidemiological situations.

(3) Unusual antibiogram or other characters.

(4) As a part of quality assurance programme.

Further reading

1. Parker M.T. Enteric Infections: typhoid and paratyphoid fever In Topley and Wilson's Principles of Bacteriology, Virology and Immunology. Arnold Publications, London, 1990.
2. Suffipant Sarasombath, Sansnee Senawong: Proceedings of Second Asia – Pacific Symposium. Typhoid Fever and other Salmonellosis. South East Asia Journal of Tropical Medicine and Public Health 26, Supplement 2, 1995.
3. Ichhpujani R.L., and Bhatia Rajesh: Typhoid Fever. Top Publications, Delhi, 1997.

Chart 1: Processing of Blood in a Patient with PUO

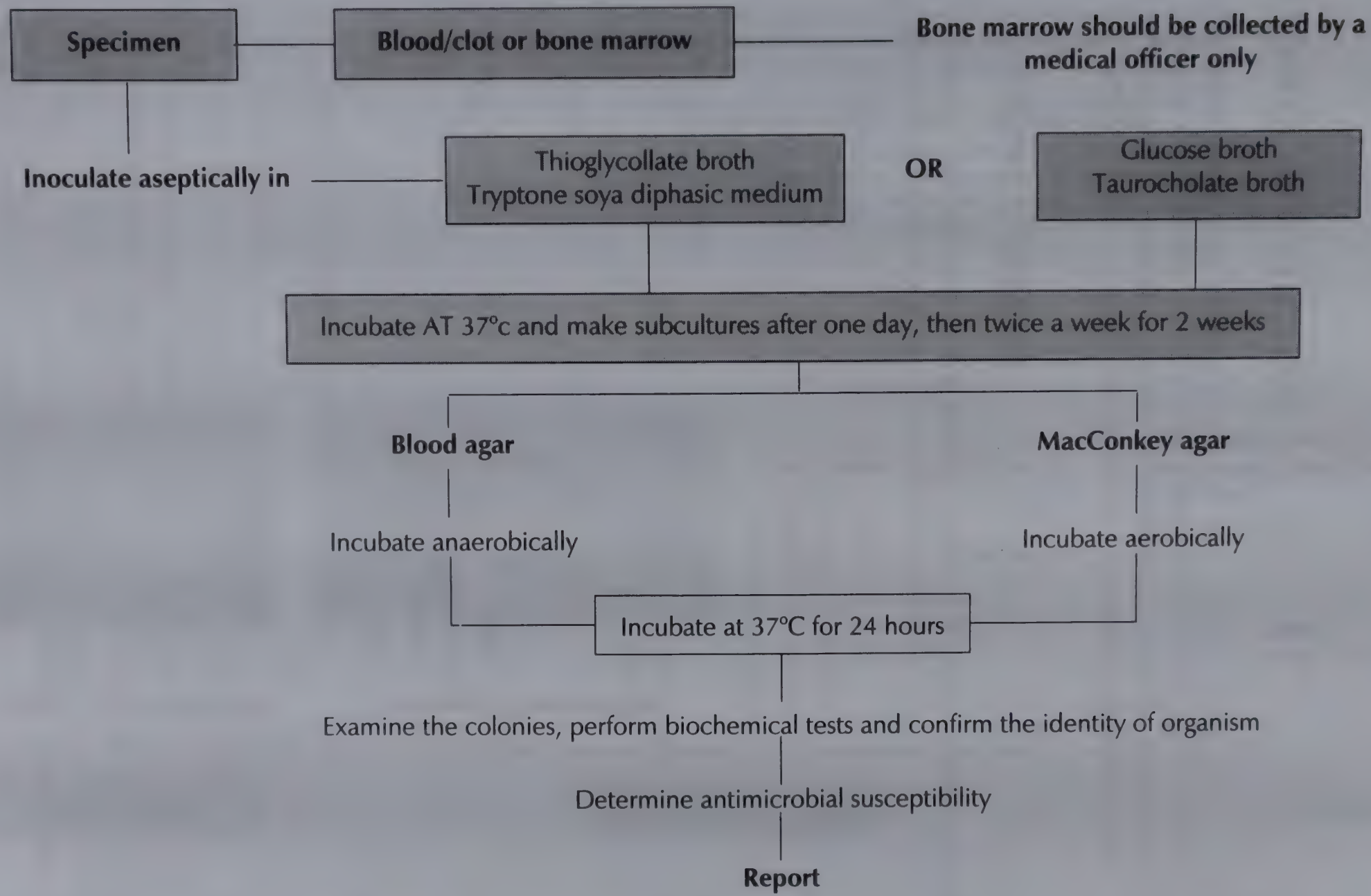
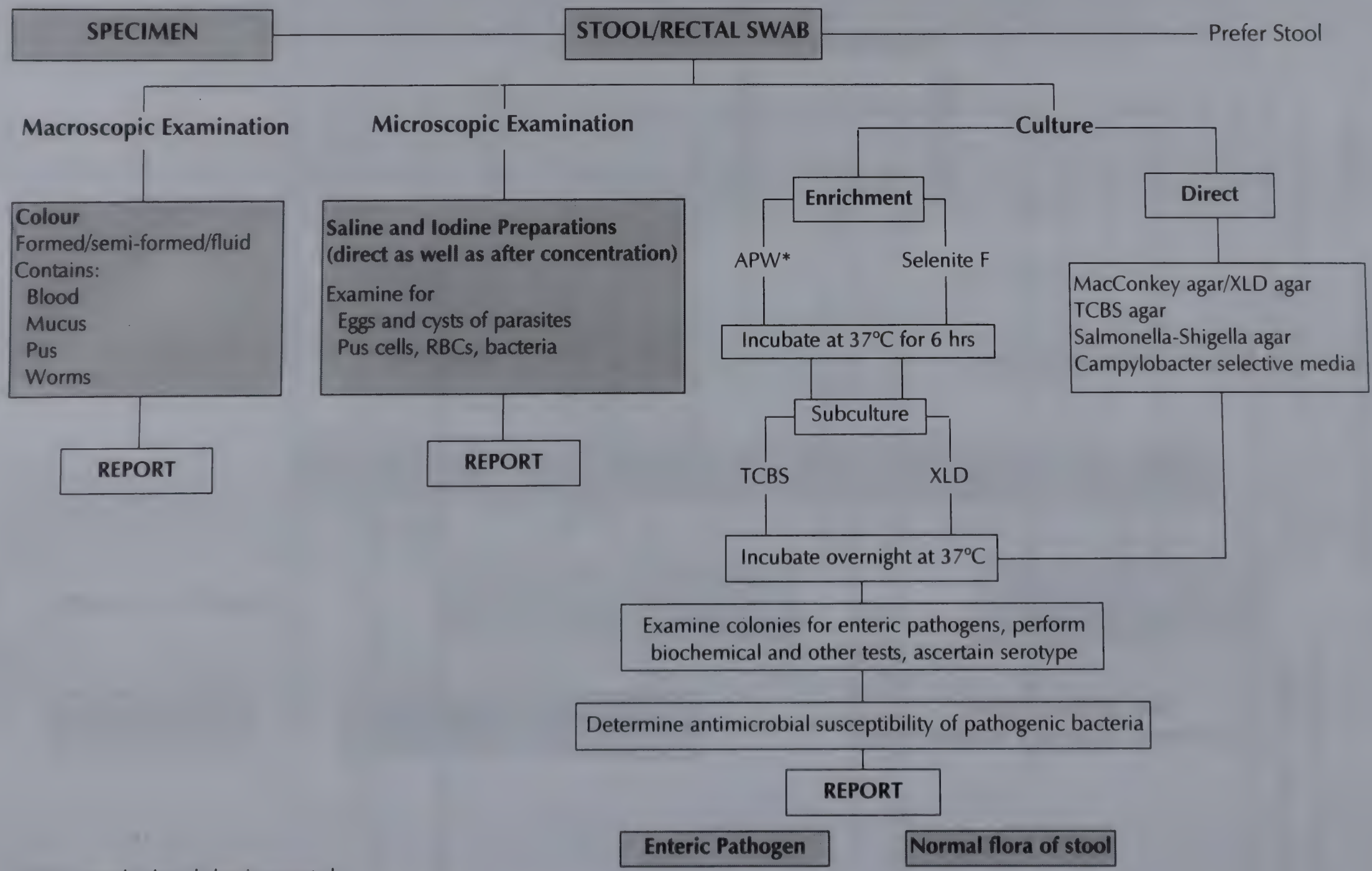

Specimen
Blood/clot or bone marrow
Bone marrow should be collected by a medical officer only
Inoculate aseptically in
Thioglycollate broth
Tryptone soya diphasic medium
OR
Glucose broth
Taurocholate broth
Incubate AT 37°c and make subcultures after one day, then twice a week for 2 weeks
Blood agar
MacConkey agar
Incubate anaerobically
Incubate aerobically
Incubate at 37°C for 24 hours
Examine the colonies, perform biochemical tests and confirm the identity of organism
Determine antimicrobial susceptibility
Report

Chart 2: Processing Stool Specimen in Cases of Typhoid Fever, Diarrhoea and Dysentery



* APW – use only when cholera is suspected.

13. Pyogenic Meningitis

Meningitis results from infection of the meninges. Pyogenic meningitis is a serious condition which can lead to brain damage and death especially in younger children. Early diagnosis is hence essential. Diagnosis is best established by laboratory examination of the cerebrospinal fluid. However, therapy should not be dependent on laboratory results.

Causative organisms

Certain bacterial pathogens tend to be characteristically associated with the age of the patient or certain disease situations. These are listed as follows:

Upto 2 months	*Escherichia coli* Group B streptococci *Neisseria meningitidis* *Listeria monocytogenes*
Less than 10 years	*N.meningitidis* *Streptococcus pneumoniae* *Haemophilus influenzae*
Adults	*N.meningitidis* *S.pneumoniae*
Elderly	*S.pneumoniae* *Gram negative bacilli* *N.meningitidis* *L.monocytogenes*
After head injury/middle ear disease	*S.pneumoniae*
After neurosurgery	*Staph. aureus* Enterobacteriaceae members

The Gram stained smear made directly from the CSF or from the centrifuged deposit can reveal not only the Gram character of the causative organism, but can also clinch the diagnosis in some cases. Presence of Gram positive lanceolate diplococci or in short chains is suggestive of *S.pneumoniae* while presence of Gram negative bean shaped, plano-convex diplococci are indicative of *N.meningitidis* infection. Similarly, Gram positive cocci in chains is suggestive of streptococci.

Collection, storage and transportation of specimens

Cerebrospinal fluid and blood should be collected from all the cases with meningitis (see Chapter 2 for details of collection of specimens). Petechial fluid can be another specimen in cases with meningococcal meningitis.

Cerebrospinal fluid (CSF)

- Collect CSF before commencement of antimicrobial therapy. However, treatment must not be delayed pending lumbar puncture.
- It is preferrable to make a smear for direct demonstration of organisms from CSF at the time of collection itself.
- Do not refrigerate the specimen.
- Culture as rapidly as possible, preferably by bedside.
- In case of delay incubate at 37°C.
- Do not expose the specimen to sunlight.
- Handle it as a precious specimen since a repeat is extremely difficult to obtain.

Blood

Blood culture can enhance the chances of diagnosis for those organisms which follow haematogenous route to reach the meninges.

- Collect 5-10 ml of blood under aseptic conditions and inoculate into at least 100 ml of blood culture medium.
- Extended culture is preferred and the inoculated media should not be discarded before seven days of incubation.

Petechial fluid

Petechial lesions, if present, may be gently irrigated by injecting 0.2 ml of sterile saline solution using a small syringe with a fine needle and the fluid collected for making smear and doing culture examination. This is particularly important for diagnosis of meningococcal disease.

Storage

Specimen should be cultured as soon as possible after the receipt. Cells disintegrate and a delay may produce a cell count that does not reflect the clinical situation of the patient. The specimen should be stored at 37°C till microscopy and bacterial cultures have been performed after which it can be refrigerated for further use.

Examination of CSF

An examination of CSF involves the following:

- Macroscopic examination for colour, turbidity and clot.

- Microscopic examination for polymorphs, lymphocytes, RBCs.
- Examination of Gram stained smear.
- Biochemical tests for protein and glucose.
- Culture for suspected pathogens.
- Latex agglutination test for antigen detection, if feasible.

Abnormalities associated with pyogenic meningitis

- Pus-like or milky CSF due to raised WBC count.
- Raised intracranial pressure.
- Reduced glucose concentration.
- Elevated protein concentration.

The CSF should arrive still warm and either be examined immediately or placed in an incubator for examination within an hour. If delay is anticipated either in transportation to the laboratory or for examination, CSF should be divided into two containers: one in a plain Bijou bottle and the other in a bottle having a few drops of glucose broth. In the laboratory CSF from the plain bottle can be used for making smears for staining whereas cultures are done from containers having CSF in glucose broth.

Macroscopic examination

- Note the colour and the presence of turbidity, deposit or clot

Clear and colourless	Normal
Clear with Tyndall effect *(sparkling appearance against incident light)*	High protein content
Clear yellowish	Old haemolysis
Clear red	Fresh haemolysis
Turbid blood-stained	Haemorrhage
Turbid white	High cell or protein content
Turbid clot *(after storage for 12-24 hours)*	Fibrin clots

- When there is no clot, count the number of white blood cells per cubic ml of fluid using a white cell counting chamber.
- Transfer about 2 ml of fluid to sterile centrifuge tube and centrifuge at 3000 rpm for five minutes.
- Transfer the supernatant fluid to a clean container for chemical examination.

Microscopic examination

- Stain one smear with Gram stain and search for bacteria under the microscope.
- Give initial report on the basis of observations.

Biochemical and cytological examination

Sugar and protein levels should be estimated and cytochemical examination be undertaken for the presence of polymorphonuclear cells.

Culture

- Centrifuge one part of CSF specimen at 2000 rpm for 5 minutes.
- Seed the deposit on chocolate agar plate.
- Allow the inoculum to dry before spreading to minimize any antibiotic effect which may be present.
- Incubate in air plus 5-10% carbon dioxide.
- Incubate remainder of the fluid which has not been centrifuged overnight. When the primary culture is sterile it is sometimes possible to recover the pathogen by repeating the subcultures from the incubated fluid next day.

The processing can be as per the flow-chart given as Chart 1 in this Chapter. For identification of *Streptococcus pneumoniae*, optochin sensitivity testing on primary culture is useful for rapid identification, whereas staphylococcal streak technique is useful for identification of *Haemophilus influenzae*.

Optochin sensitivity test

This test is performed with commercially available discs impregnated with 5 µg of optochin. The test is performed as under:

- Using the inoculating loop or wire, select 3-4 well isolated colonies of the organism to be tested and streak that on one half of a culture plate of sheep blood agar in an area around 3 cm square.
- Place an optochin disc in the upper third of the streaked area and press the disc with flammed forceps so that disc adheres to the agar surface.
- Incubate the plate at 35°C for 18 to 24 hours in a candle jar.
- Alpha haemolytic streptococci showing a zone of inhibition of 14 mm or more around a 6 mm disc can be presumptively identified as *Streptococcus pneumoniae*.
- Use *S. pneumoniae* as positive control and *Strept. viridans* as negative controls.

Similarly placing paper discs containing X,V factors or streaking *Staphylococcus aureus* culture across the plate will enhance the ability to recover *Haemophilus influenzae* organisms.

Staphylococcus streak technique

- Subculture a colony of the suspected *Haemophilus spp.* on a sheep blood agar plate and streak it as a lawn.

- Using an inoculum wire, make a streak of *S.aureus* through the inoculum of possible *Haemophilus spp*.
- Incubate at 35°C for 18-24 hours in 5-10% CO_2 in a candle jar.
- Tiny, moist colonies appear within the haemolytic area adjacent to the staphylococcal growth. This method is used for the isolation and presumptive identification of *Haemophilus*.

Immunological tests

Tests are now available to detect antigen in the CSF which are feasible at peripheral as well as intermediate laboratories. These are:

- ELISA
- CIEP
- Coagglutination
- Latex agglutination

Of all these tests latex agglutination is rapid, easier to perform, sensitive, and specific. Kits are commercially available and test can be performed under field conditions.

Latex agglutination test

The commercially available kits contain reagents for detecting following antigens:

- *Neisseria meningitidis* serogroup A
- *Neisseria meningitidis* serogroup B
- *Neisseria meningitidis* serogroup C
- *Streptococcus pneumoniae*
- *Haemophilus influenzae b*

Procedure for latex agglutination

- Heat CSF specimen in a water bath at 80-100°C for 5 minutes.
- Centrifuge at 2000 rpm for 10 minutes.
- Use supernatant.
- Thoroughly shake latex suspension.
- In the corresponding field of slide dispense 1 drop of latex suspension and then one drop of the supernatant of the CSF specimen.
- Mix with a stirring stick (use one stick per specimen).
- Rotate the slide and read the results within 2 minutes.

Reading

➢ **Negative reaction**

Milky suspension
(disregard any granules that may occur with *S.pneumoniae*)

➢ **Positive reaction**

Distinct and rapid agglutination occurring within 2 minutes
(usually in 30 seconds)

Interpretation

Agglutination with one of the latex reagents indicates presence of corresponding antigen in CSF and is diagnostic.

Controls

Periodically check that none of the five latex reagents agglutinate in the presence of 0.15 mol/L NaCl solution and that each of these latex reagents do agglutinate with respective positive controls.

Important points in diagnosis

- ➢ Acute bacterial meningitis is a life threatening condition and specimen deserves immediate processing.
- ➢ Diagnosis is best established by laboratory examination of CSF.
- ➢ The management of the patient should base on immediate cell-count and Gram stain smear results.
- ➢ The CSF should be examined at the earliest, as on storage cells disintegrate and produce a cell count that does not reflect the true clinical situation.
- ➢ Perform cell count on the uncentrifuged CSF specimen.
- ➢ When a clot is present in CSF, it invalidates the CSF cell count.
- ➢ If there is an insufficient volume of sample for carrying out all the investigations, prioritize the tests as per the medical advice.
- ➢ Notify to the public health authorities isolation/detection of any epidemic prone pathogens (e.g.*N.meningitidis*).

Antimicrobial susceptibility testing

For individual patient management, physicians hardly wait for antimicrobial susceptibility reports, as the treatment is usually initiated with more than one antimicrobial agents. Nevertheless, the laboratory must have its policy regarding testing for antimicrobial susceptibility. For organisms such as *Neisseria meningitidis* and beta haemolytic streptococci with predictable susceptibility patterns, there is no need of performing

antimicrobial susceptibility testing.. For all other organisms antimicrobial susceptibility tests be done as per standard methodology. (Refer Chapter 7).

Quality assurance

- Follow manufacturer's instructions for media and diagnostic kits.
- Follow standard QA procedures for laboratory equipment and test methods as outlined in chapter 9.

Biosafety

- Wash hands particularly after collection and handling of the clinical specimen.
- Use leak proof containers in sealed plastic bags for transportation of the sample.
- Perform all work on suspected isolates of *N.meningitidis* which is likely to generate aerosols in a safety cabinet.

Referral

- β-haemolytic streptococci, *H.influenzae* and *S.pneumoniae* for serotyping.
- *N.meningitidis* for strain characterisation and antimicrobial susceptibility testing.
- Isolates associated with outbreaks.
- Organisms with unusual or unexpected resistance patterns.
- In clinically suspected meningococcal disease outbreaks where diagnosis has not been possible.

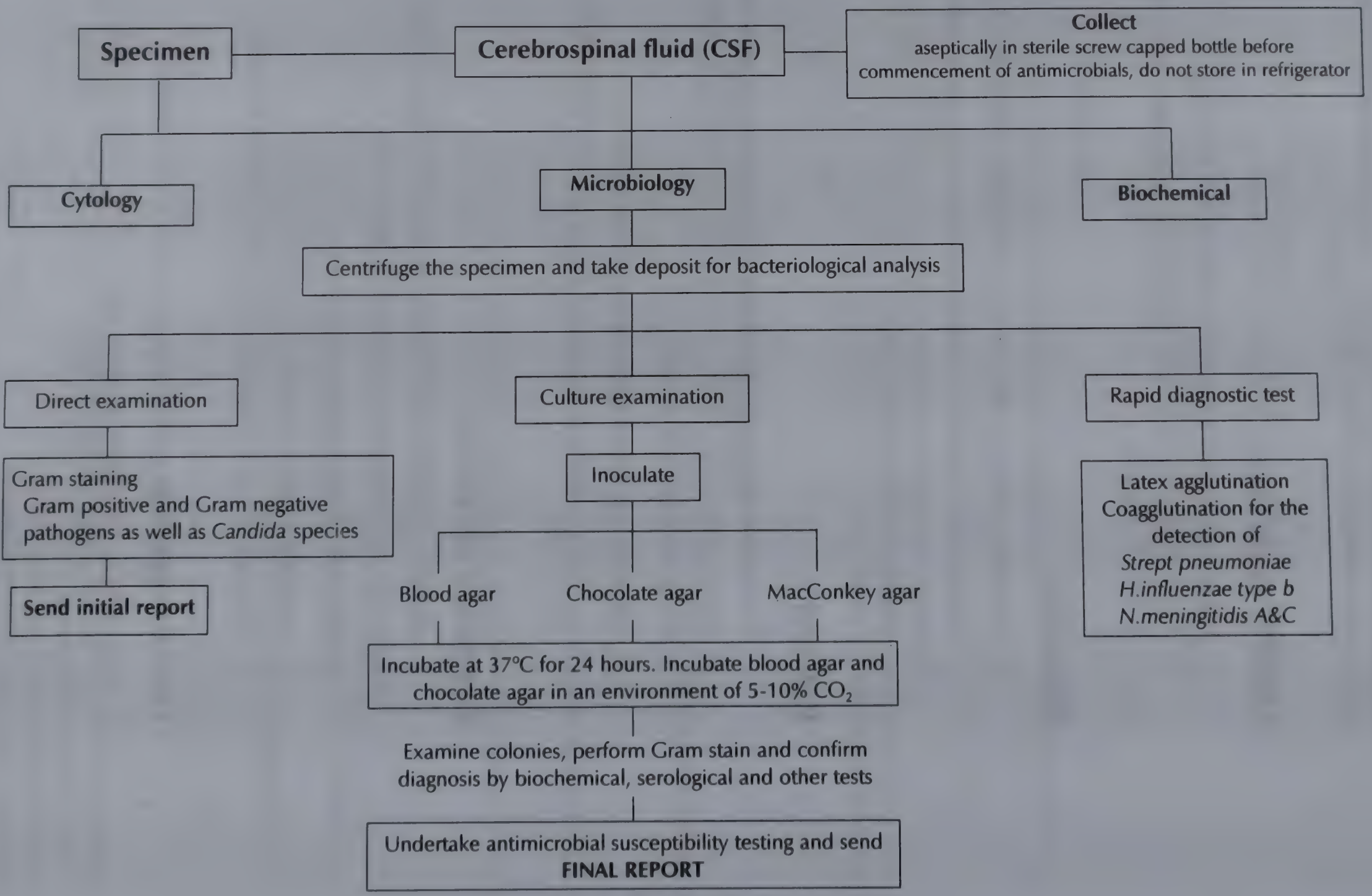
Reporting of results

- Report the appearance of CSF and also the presence or absence of blood clot.
- Report number of polymorphs and lymphocytes per litre.
- Report on organisms detected on Gram stain.
- Report the organism isolated or report absence of growth.
- Report antimicrobial susceptibility test results as clinically indicated.

Further reading

1. Daly J. Seskin KC and Pezzlo M. Processing and interpretation of cerebrospinal fluid. In Isenberg HD (ed.) Clinical Microbiology Procedures Hand book, ASM, Washington DC, 1992 Vol.1, Section 1.9.

Chart 1: Diagnosis of Acute Pyogenic Meningitis


Specimen
Cerebrospinal fluid (CSF)
Collect
aseptically in sterile screw capped bottle before commencement of antimicrobials, do not store in refrigerator
Cytology
Microbiology
Biochemical
Centrifuge the specimen and take deposit for bacteriological analysis
Direct examination
Culture examination
Rapid diagnostic test
Gram staining
Gram positive and Gram negative pathogens as well as Candida species
Inoculate
Latex agglutination
Coagglutination for the detection of
Strept pneumoniae
H.influenzae type b
N.meningitidis A&C
Send initial report
Blood agar
Chocolate agar
MacConkey agar
Incubate at 37°C for 24 hours. Incubate blood agar and chocolate agar in an environment of 5-10% CO_2
Examine colonies, perform Gram stain and confirm diagnosis by biochemical, serological and other tests
Undertake antimicrobial susceptibility testing and send
FINAL REPORT

14. Dysentery

Dysentery is a clinical entity, characterized by frequent passage of blood-stained mucopurulent loose stools. Aetiologically, it is divisible into two main categories:

- Amoebic dysentery
- Bacillary dysentery

Bacillary dysentery is mainly caused by members of the genus *Shigella* while amoebic dysentery is caused by *Entamoeba histolytica*. Bacillary dysentery usually occurs in the form of outbreaks while amoebic dysentery presents as sporadic cases. The diagnosis is based upon microscopic examination and isolation of the organism.

Symptoms of *Shigella* infection include abdominal cramps, tenesmus, frequent small volume bloody stools and mucoid discharge. The infection is transmitted from person to person and infectious dose is very small. Outbreaks are associated with low standards of sanitation and hygiene.

Collection of faeces

Faecal sample or faecal swabs are preferred. If properly collected, rectal swabs also yield good results. Observe the following precautions while collecting the faecal specimen:

- Collect during the early stage of the disease and before antimicrobial therapy has been initiated.
- Collect in a clean, preferrably sterile, screw-capped container.
- Process the collected stool specimen within two hours. If delay of more than two hours is anticipated, inoculate the specimen in a transport medium such as Cary and Blair transport medium.
- Collect the specimen from freshly voided stool containing blood and mucus. If the transport medium is not available samples may be collected in a container with buffered glycerol saline.
- If faecal swab is to be collected, dip a sterile swab stick in the faeces and put the swab in a sterile tube.
- For collection of rectal swab, cotton tipped swabs should be used. Ensure that the cotton fits tightly on the stick. Moisten the swab in sterile normal saline, insert through rectal sphincter, rotate and withdraw. Examine the swab for faecal staining.

Specimen of faeces should be transported to the laboratory and processed at the earliest, as *Shigella spp*. may not survive the pH changes that occur in faeces specimens which are not properly delivered to the laboratory even if refrigerated. Cary and Blair transport medium which is available at most of the intermediate laboratories, is a satisfactory transport medium.

Macroscopic examination

➢ Examine the stool specimen for consistency (liquid, semisolid or formed); colour (white, yellow or black), atypical components (mucus, blood, parasites etc).

Microscopic examination

➢ Examine microscopically for parasites, leucocytes and RBCs by making saline and iodine preparations.

➢ Prepare a faecal suspension by suspending the faeces from the rectal or faecal swab in a tube containing 1 ml of sterile saline per swab. Wash the swabs thoroughly in the saline by swirling the tube and rotating the swab against the side of the tube to express the fluids.

The distinguishing features between bacillary and amoebic dysentery are given in Table 1.

Table 1: ***Differences between bacillary and amoebic dysentery***

Feature	Bacillary dysentery	Amoebic dysentery
Pus cells	Numerous	Scanty
Red blood cells	Discrete	In clumps
Eosinophils	Absent or rare	Present
Macrophages	Present and show ingested RBCs	Absent
Charcot Leyden crystals	Absent	Present
E.histolytica	Absent	Trophozoites +
Bacteria	Scanty, non motile	Numerous, motile
Growth on MacConkey agar	Positive for *Shigella*	Negative

Isolation of organism

➢ If bacillary dysentery is suspected, attempt to isolate *Shigella* from stool specimen.

➢ Inoculate the stool specimen/rectal swab on MacConkey agar or deoxycholate citrate agar.

➢ Incubate aerobically overnight at 35-37°C.

- Look for typical colonies on these media. Pale, yellowish, non-lactose fermenting on MacConkey agar and pale, translucent and smaller colonies on DCA and pink colonies on XLD are suggestive of shigellae.
- Perform oxidase test which is negative for shigellae.
- Check motility of the isolate by a hanging drop preparation. Shigellae are nonmotile organisms.
- Identify *Shigella* genus on the basis of important biochemical reactions which are urease, indole, fermentation of glucose, mannitol and sucrose.
- If the organism is biochemically *Shigella spp.*, report the same as such.
- A simple flow chart for identification of various *Shigella spp.* is given as Chart 1.

Cautions

- While looking for trophozoites of *E.histolytica*, examine freshly passed stool sample preferably within 30 minutes of collection from the patient and not received in the laboratory.
- Take care not to mistake macrophages for the trophozoites of *E.histolytica*.

Biosafety

- Transport the specimen in a leak- proof container in a sealed plastic bag.
- Perform the examination/isolation work in at least biosafety level 2.
- Observe good laboratory practices, particularly hand-washing after processing the specimen.

Referral

- For serotyping
- Confirmation of the isolate in case of outbreak
- In the event of unusual or unexpected antibiotic resistance

Antimicrobial sensitivity testing

With the emergence of drug resistance in *Shigella spp.*, it is advisable to perform antimicrobial susceptibility test, particularly in an outbreak situation. Follow the procedure for antimicrobial suspectibility testing outlined in Chapter 7.

Reporting of results

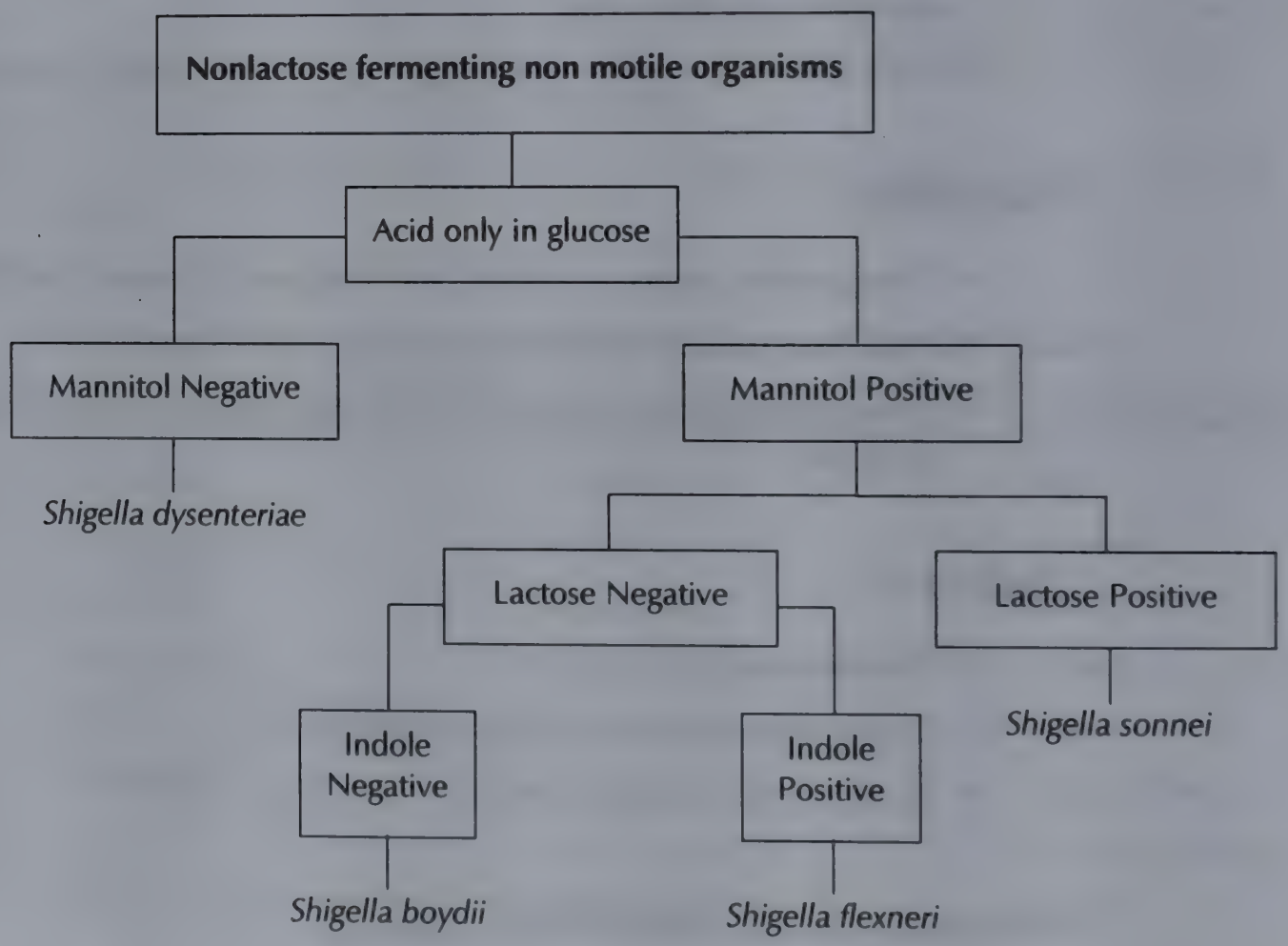
- Report on the presence of blood and/or mucus.

- Report on the presence of ova/cysts and the trophozoites.
- Report about the presence of pus cells, eosinophils, macrophages, CL crystals and bacteria.
- Report about the presence of bacterial species particularly Shigella.
- Report about the antibiogram.

Quality assurance

- Observe quality assurance procedures covering all the pre-analytical, analytical and post-analytical aspects.
- Follow the quality assurance procedures for media, reagents and tests as outlined in Chapter 9.

Chart 1: *Biochemical identification of* Shigella *spp.*



Further reading

1. PHLS Standard Operating Procedures: Investigation of faeces specimen for bacterial pathogens B.SOP 30, 1998.
2. Pedler SJ and Orr KE Examination of faeces for bacterial pathogens J Clin Pathol 43,410-415:1990.

15. Gonorrhoea

Gonorrhoea is a common sexually transmitted disease which is caused by *Neisseria gonorrhoeae*. This is transmitted between individuals by direct, usually sexual contact. In males the most common manifestation of infection is acute urethritis characterized by abrupt onset of dysuria and a purulent urethral discharge. The primary site of urogenital gonorrhoea in females is the endocervix. Infection may be accompanied by a purulent vaginal discharge, menstrual irregularities and dysuria. *N.gonorrhoeae* is highly susceptible to adverse environmental influences such as temperature extremes and drying and it does not survive long outside its natural host which is man. The diagnosis of the disease in the laboratory is based upon:

- ➢ Direct examination of the Gram stained smear from the pus. It has high sensitivity and specificity (over 90% in males) for the diagnosis.
- ➢ Culture : isolation and identification.

Causative organism

N.gonorrhoeae is a Gram negative kidney-shaped diplococcus with slightly concave adjacent surfaces. In smears of exudates, diplococci are often seen inside polymorphonuclear leucocytes.Good clinical specimens will have numerous polymorphs which in adequate specimen may also show epithelial cells. A smear is labelled as positive when it shows many intracellular Gram negative diplococci.

Specimen collection

For diagnosis of gonorrhoea, appropriate sites for specimen collection depend to some extent on the age, sex and sexual practices of the individual and the clinical features of the infection. The commonest sample is pus from genitourinary passage.

Collection of specimen from males

- ➢ Use bovine serum albumin coated swabs since plain cotton is inhibitory to gonococci. These can be prepared by dipping cotton swabs in 20% bovine albumin solution, dried at 37°C and sterilized by autoclaving.
- ➢ Collect the sample in the morning before the patient has voided urine.
- ➢ If necessary, clean the meatus with a swab moistened in normal saline or plain lukewarm water.
- ➢ Exert a slight pressure on penis so that a drop of pus appears at meatus.

- Remove the pus with a sterile inoculating loop or apply directly to a clean glass slide.
- If no pus appears, insert the sterile loop approximately 2.5 cm up the urethral canal to obtain a specimen or obtain by prostatic massage.
- Prepare smears on two different slides by spreading the smear as thinly as possible.
- Anorectal specimens are collected by inserting a swab 4-5 cm into the anal canal.

Collection of specimen from females

- Take the help of a specialist for collecting sample by a swab from the cervical canal.
- In case of chronic gonorrhoea, the specimen should be taken just before or after the menstrual period.
- Insert the sterile swab 2-3 cm into the cervical canal and rotate it for 5-10 seconds to permit absorption of the exudate.
- Before inoculating the culture or transport medium, it is desirable to prepare a smear for microscopy. Prepare the slides in duplicate by rubbing the pus swabs over a clean slide in a thin film. To obtain a thin homogenous film, roll swab onto a clean slide and allow the smear to air-dry.

Staining the smear and examination

- Fix the smear by passing through the flame.
- Stain with Gram stain.
- Examine the stained smear under 100x oil immersion by paying special attention to the edges of the smear, where the smear is the thinnest and it is convenient to identify the gonococci, if present.

Look for the following under the microscope:

- pus cells with bright pink nuclei and colourless cytoplasm. In gonococcal urethritis usually there are > 10 pus cells per oil immersion field.
- gonococci which are oval, kidney shaped, Gram negative and arranged in pairs, and are present intracellularly or extracellularly.
- slide should be examined for atleast 2 minutes before declaring it negative for Gram negative intracellular diplococci.

The results of the microscopic examination are interpreted as under:

- many leucocytes, a few RBCs and epithelial cells and many Gram negative intracellular diplococci would indicate ***gonococci present in the specimen.***
- many leucocytes, a few RBCs and epithelial cells but no Gram negative diplococci present within cells but a few are present extracellularly : ***gonococci suspected.***
- many leucocytes, a few RBCs and epithelial cells, no Gram negative diplococci : ***negative for gonococci.***

Specimen transportation for culture

When the diagnostic facilities do not exist locally and the specimens need to be transported, it can be done in one of the following ways:

- If standard culture media are available at the collection site, inoculate the specimen directly, place in an atmosphere containing 5% CO_2 at 37°C. Transport this to laboratory.
- If culture media are not available locally, transport medium can be used. Even with the use of transport media, results are not satisfactory if transit takes more than 48 hours. The swabs can be inoculated into a non-nutrient transport medium as modified Stuart Transport medium or charcoal free Amies Transport medium. These can be transported at room temperature. Isolation rate is approximately 100% within 12 hours and more than 90% within 24 hours, although the number of colonies decreases markedly.

If no transport medium is available, use a Pasteur pipette to collect the specimen as described below:

- Draw the pus specimen into a sterile Pasteur pipette plugged with cotton wool at the top. Heat seal the tip.
- Place the pipette as it is in a sterile tube, padded and plugged with cotton wool.
- This method also preserves the specimen at room temperature for 6 hours.

Culture

Gonococci are fastidious in their growth requirements. Most of the times these are present alongwith normal flora. The culture media used are, therefore, both enriched and selective. For routine culture, GC agar base medium of good quality with 9% saponin lysed horse/sheep blood and inhibitors (vancomycin to suppress the growth of Gram positive, colistin to suppress Gram negative organisms, nystatin to suppress yeast multiplication and trimethoprim is added to inhibit swarming by *Proteus* spp.).

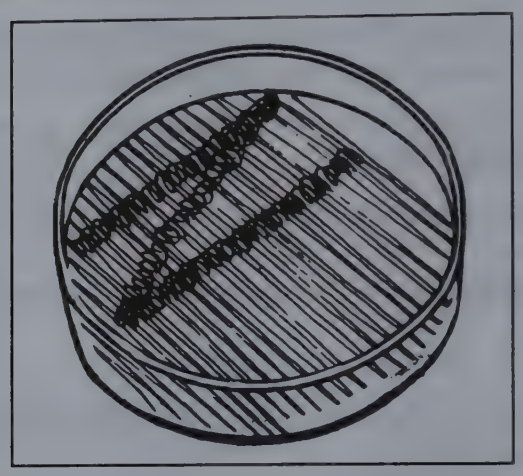
The culture is done as follows:

- Inoculate the swab onto a 1 cm circular area of the agar plate by rubbing the whole surface of the swab.
- Streak out the inoculum on the whole plate with the nichrome wire loop, bent at an angle of 30°. The inoculum is spread out as shown in Fig 1 to obtain isolated colonies.
- Incubate in a humid environment of air containing 3-5% CO_2. This can be obtained by a candle extinction jar and by placing wet paper towels in the incubator.
- Incubate for a minimum of 48 hours before discarding the plates.
- Examine the plates after 18-24 hours of incubation.

HEALTH CELL
LIBRARY
AND
DOCUMENTATION
UNIT

- Typical colonies are 0.5-1 mm in diameter, smooth, grey, translucent and slightly raised.
- A presumptive diagnosis can be made by performing Gram stain, oxidase test and superoxol test. For definitive identification the isolate can be sent to reference laboratory.
- Gram staining is performed on single suspect colony from the primary culture as per method described earlier and looking for Gram negative diplococci.

Figure 1: *Inoculation of culture plate*



Oxidase test

- Moisten a small piece of filter paper with 2-3 drops of oxidase test reagent and keep it in an inverted petri dish lid.
- Using a wooden applicator or the tip of a glass pipette, streak a suspect colony on the surface of a moistened filter paper.
- Development of purple colour within five seconds indicates a positive test.

Superoxol test

It can be performed either on a slide or on a plate.

- Emulsify a few colonies of the culture to be tested with a loop in a drop of 30% w/v hydrogen peroxide.
- Immediate (within 1-2seconds) production of bubbles is defined as positive superoxol test.

Presumptive identification

Biochemical identification tests are neither feasible nor desired at the peripheral and intermediate laboratories for the identification of gonococci. A presumptive identification of gonococci isolated from urogenital tract is based on followings:

- Typical colony morphology
- Gram negative diplococci

- Positive oxidase test
- Positive superoxol test

Biosafety

- Observe general biosafety measures as outlined in Chapter 8.

Quality assurance

- Observe internal quality control measures for equipment, culture media, staining and reagents as outlined in Chapter 9.
- Give due emphasis to pre-analytical and para analytical factors affecting the quality.

Reporting of results

Gram stained smear

- Many leucocytes, a few RBCs, many Gram negative intracellular diplococci-Report presumptively positive.
- Many leucocytes, a few RBCs, no intracellular diplococci but a few extracellular diplococci-Report gonococci suspected.
- Many leucocytes, a few RBCs, no Gram negative diplococci seen-Report negative for gonococci.

Culture

- Typical colony characters, Gram negative diplococci, oxidase and superoxol test positive-Report positive.

Antimicrobial susceptibility

These results can be communicated later.

Antimicrobial susceptibility testing

Refer the isolate of gonococcus to the nearest reference laboratory by inoculating it on a chocolate agar slant for determination of susceptibility to antimicrobial agents and screening for beta lactamase production. Although gonococci have become increasingly resistant to penicillin and other antimicrobial agents, the cure rates are still very high in cases of uncomplicated urogenital infections when treated with penicillin in adequate dosages.

Referral

The isolates of gonococci should be sent to the referral laboratories for the followings:

- ➢ Confirmation of the isolate
- ➢ Antimicrobial susceptibility testing
- ➢ Beta lactamase production testing
- ➢ Isolates from clusters of treatment failures

Further reading

1. Laboratory diagnosis of gonorrhoea: WHO Regional Publication South-East Asia Series No 33, 1999.
2. Edwin H Lennette, Albert Balows, Williams J Hausler, H Jean Shadomy. Neisseria and Branhamella in Manual of Clinical Microbiology (4th Ed), 176-192,1985.

16. Syphilis

Syphilis is caused by a treponeme called *Treponema pallidum*. The disease manifests as primary, secondary and tertiary stage. The primary stage is characterized by the formation of an ulcer (called chancre) on the genitalia. The diagnosis can be done by:

- Direct demonstration of *T.pallidum*
- Serological evidence

In the primary stage, the spirochaetes are abundant in the lesion while antibody may be totally absent. Serological diagnosis is the mainstay in secondary and tertiary stages.

Direct demonstration

The detection of treponemes within lesion material is a key aid to diagnosis. Dark field microscopy is the recommended method.

Collection of specimen

- Collect the specimen before the antibiotic therapy has begun.
- If multiple lesions are present, choose the youngest one available.
- Clean the surface of the lesion with saline and blot it dry.
- Gently remove the crusts of the lesions if these have already crusted.
- Superficially abrade the lesion until very slight bleeding occurs. This is done to obtain the clear serum exudate from the lesion subsurface.
- Apply gentle pressure at the base of the lesion and wipe away the first few drops of blood.
- Touch a glass slide to the clear exudate, place a cover slip on the specimen and immediately examine the slide by dark field microscopy.
- The exudate can also be removed with a capillary pipette and then transferred to a glass slide. Alternatively, the lesion material may be aspirated with a 26 gauge needle inserted at the base of the lesion after injecting a drop of saline in it.

Storage and transportation

Treponemes are very susceptible to environmental influence and are readily inactivated by heat, cold, desiccation and most disinfectants. Because of their delicate nature, it is

important to examine lesion material immediately. Storage at room temperature or refrigerator temperature even for a few hours is not recommended.

Serum samples are stored at 4°C or -20°C. A few drops of finger-prick blood sample collected on filter paper strip can be safely transported to the laboratory. The elute obtained from this can be used for testing purposes.

Examination under dark field microscope

- A microscope equipped with a good light source and a dark field condenser must be available.
- Place a few drops of immersion oil on the condenser of the dark field microscope.
- Lower the condenser slightly so that the oil is below the level of the stage.
- Place the slide on the microscope and raise the condenser until there is a good contact between the oil and the underside of the slide. Carefully avoid trapping air bubbles in the oil.
- Using the low power objective (10x) bring the specimen into focus. Centre the light in the field by adjusting the centring screws located on the condenser and focus the condenser by raising or lowering it until the smallest diameter of the light is obtained.
- Use the dry 40x objective, bring the specimen in focus and examine the slide carefully. Avoid bright day light.

Observations

T.pallidum appears white, illuminated on a dark background. It is identified by its typical morphology, size and movements. It is a thin organism with 8-14 regular highly wound deep spirals. It exhibits quick and rather abrupt movements. It rotates relatively slowly about the longitudinal axis like a corkscrew. The rotation is accompanied by twisting in the middle and is executed rather stiffly. Lengthening and shortening like an elastic expander may also be observed.

The demonstration of *T.pallidum* is diagnostic of primary and secondary syphilis.

Cautions

- Failure to find an organism in dark field microscopy does not rule out syphilis.
- Negative results may be obtained in patients who are on antimicrobial therapy or when insufficient number of organisms are present in the lesions or when the lesion is approaching natural resolution.
- Whenever the results are negative in dark field microscopy, a blood sample should always be taken for serological tests.
- Since the diagnosis is based on the motility of the organism, erroneous results may be obtained if old specimens are tested.

Reporting of results

Presence of *Treponema pallidum* like organisms in dark field smear confirms diagnosis of primary syphilis.

Absence of *Treponema pallidum* like organisms does not rule out syphilis.

Susceptibility testing

Treponema pallidum continues to be sensitive to penicillin/erythromycin. There is no need for antimicrobial susceptibility testing.

Serological evidence

VDRL test

This is a slide flocculation test which detects non-treponemal (reagin) antibodies in the serum. With minor modifications it can be done on cerebrospinal fluid also.

VDRL on serum

Heat clear serum at 56°C for 30 minutes immediately before the test is to be done. Serum to be tested more than four hours after being heated should be reheated at 56°C for 10 minutes.

Antigen

This can be procured commercially. It is an alcoholic solution containing 0.03% cardiolipin, 0.9% cholesterol and purified lecithin sufficient to produce standard reactivity. Buffered saline is also supplied alongwith the commercial preparation for preparing antigen suspension. If buffered saline is not supplied with the antigen, same should be prepared in the laboratory.

Procedure

Preparation of suspension

- Pipette 0.4 ml of buffered saline solution to the bottom of a 30 ml ground glass or screw cap stoppered bottle.
- Add 0.5 ml antigen directly onto the saline solution within 6 seconds while continuously, but gently, rotating the bottle on a flat surface.
- Blow last drop of antigen from pipette without touching pipette to saline solution.
- Continue rotation of bottle for 10 more seconds.

- Add 4.1 ml buffered saline solution.
- Place cap on bottle and shake vigorously for approximately 10 seconds and leave it in a dark place.
- The antigen suspension thus prepared should be used within one day and only after half an hour of preparation.

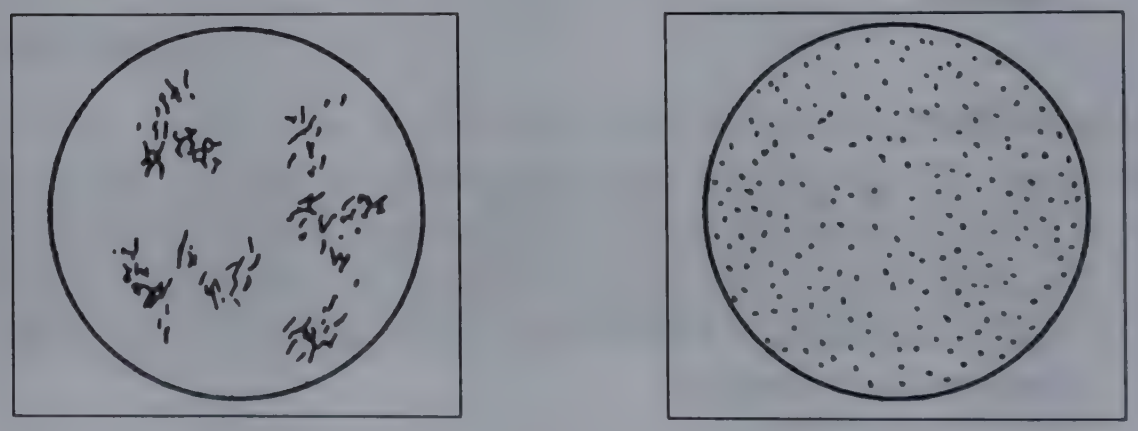
Checking of antigen delivery needle

- The number of antigen particles per microscopic field is determined by the size of the drop of antigen suspension. Hence, the needle used should be checked every day.
- Use a 18 or 19 gauge needle, with cut tips (without bevel) or 23 gauge with bevel and attach it to a 1 ml syringe to ensure that 60 drops are obtained from 1 ml of antigen suspension.
- Hold the syringe in such a way that the needle tip or bevel is down and the dropping surface is horizontal. In case the needle has cut tips, the syringe need to be held vertically.

Checking of antigen suspension

Each preparation of antigen suspension should first be examined by testing with known positive or negative serum controls. The antigen particles appear as short rod forms at magnification of about 100x. Aggregation of these particles into large or small clumps is interpreted as degrees of positivity (Fig 1).

Figure 1: *Reactive VDRL* *Non-reactive VDRL*



Procedure

Qualitative serum slide test

- Pipette 0.05 ml of each heated serum into a ring on VDRL glass slide.
- Set up pretested control sera to give reactions of varied degrees as a control and guide for reading.
- Add one drop (0.016 ml) antigen suspension to each.
- Rotate slides for 4 minutes. Rotation can be on a VDRL shaker or manually. If done manually, the movement should roughly circumscribe a two inch diameter circle 120 times per minute.
- Read test carefully after rotation and record.

Recording results

No clumping or slight clumping	Non-reactive
Small clumps	Weakly reactive
Medium and large clumps	Reactive

Report results of qualitative test only as reactive (R), weakly reactive (WR) or non reactive (NR).

Quantitative serum slide test

Serum dilutions

- Pipette 0.5 ml of freshly prepared physiological saline into each of five or more test tubes.
- Add 0.5 ml of heated serum to tube No 1, mix well and transfer 0.5 ml to tube No 2.
- Continue mixing and transferring until the last tube contains 1 ml. Discard 0.5 ml from this. It would give dilutions of 1:2, 1:4, 1:8 onwards.
- Test each serum dilution as described under qualitative serum test.

Reading and recording

Read test microscopically at 100x as described under qualitative procedure and record the titre as the reciprocal of the serial dilution that produces a reactive reaction. Record the titre as reactive and add the titre in dilutions e.g. VDRL reactive, 16 dilution.

VDRL on CSF

In testing cerebrospinal fluid for reagin antibodies the antigen is first sensitized by mixing and gently rotating it with equal amount of 10% sodium chloride solution; the needle is selected in such a way that 1 ml gives 100 drops and after mixing with 0.05 ml of CSF slides are rotated for 8 minutes. The reporting and recording is similar to that for serum samples.

Presently rapid plasma reagin (RPR) test is widely recommended for use in field studies. The test can be carried out on unheated serum or plasma and read with naked eyes. The reagents are commercially available and are economical.

Biological false positives in VDRL

- Systemic lupus erythematosus
- Malaria
- Lepromatous leprosy
- Infectious mononucleosis
- Tropical eosinophilia

- Relapsing fever
- Hepatitis
- Rheumatoid arthritis
- Collagen disorders
- Severe trauma
- Coronary artery disease
- Repeated blood loss
- Menstruation
- Vaccination
- Pregnancy
- Haemolytic anaemia
- Heroin addiction
- Tissue regeneration
- Upper respiratory infection
- Certain antihypertensive drugs

Quality assurance in VDRL testing

- Use known reactive and nonreactive sera for both qualitative and quantitative tests to validate the results.
- Practice the internal quality control measures as outlined in Chapter 9. This should cover all aspects of diagnosis comprehensively.
- The microscopist reporting on presence of *T.pallidum* is dark field microscopy should be trained.

Referral

(1) To rule out Biological False Positive VDRL results sample can be sent to a reference laboratory for specific tests.

(2) For quality assurance programme.

Biosafety

- Treponemal lesions frequently exude material that is infectious. For this reason gloves must be worn by the examiner.
- Disinfect slides and equipment used for obtaining smears for dark field microscopy. 70% ethanol is very effective for this purpose.
- Wash hands with soap and water after handling the specimen.

- Use safety pipetting devices for delivering the serum for VDRL testing.
- Before examination of clinical material, skilled use of the techniques can be learned by using nonpathogenic treponemes taken from the gum margins of healthy people.

Further reading

1. Larsen SA, Siener BM, Rudolph A.H.: Laboratory diagnosis and interpretation of tests for syphilis. Clin. Microbiol. Rev. 8: 1-21, 1995.
2. Hart G: Syphilis tests in diagnostic and therapeutic decision making. Ann. Intern Med. 104, 368-376, 1986.
3. Miller, J.N.: Value and limitations of non-treponemal and treponemal tests in the laboratory diagnosis of syphilis. Clin. Obstet. Gynaecol. 19: 191-203, 1975.

17. Tuberculosis

Tuberculosis is caused by *Mycobacterium tuberculosis* which is an acid fast bacillus (AFB). The highest priority for tuberculosis (TB) control is the identification and cure of infectious cases i.e. patients with sputum smear positive pulmonary TB. The highest priority in the diagnosis of TB is thus given to sputum microscopy. The culture of *Mycobacterium tuberculosis* may only be feasible at a few of the intermediate laboratories.

Morphology of *M.tuberculosis*

Acid fast bacilli are approximately 1-10 μ long, slender, rod-shaped bacilli which may be curved or bent. These may be granular, isolated, in pairs or in groups. Stained bacilli may present a beaded appearance.

Microscopy of sputum

Diagnosis of pulmonary TB by sputum microscopy is simple, easy, inexpensive, rapid, technically not very demanding and more reliable than X-rays. The purpose of the sputum microscopy is two fold (a) diagnosis of the patients with infectious tuberculosis (b) monitoring the progress. For diagnosis, 3 sputum examinations are performed (spot, morning, spot) and for follow up 2 sputum examinations (morning, spot) are performed.

Collection of sputum sample

- ➢ Select a good wide-mouthed sputum container, which is disposable, made of clear thin plastic, unbreakable and leak proof material.
- ➢ Give the patient a sputum container with the laboratory serial No. written on it. Show the patient how to open and close the container and explain the importance of not rubbing off the number written on the side of the container.
- ➢ Instruct the patient to inhale deeply 2-3 times, cough up deeply from the chest and spit in the sputum container by bringing it closer to mouth.
- ➢ Make sure the sputum sample is of good quality. A good sputum sample is thick, purulent and sufficient in amount (2-3 ml).

Give the patient another container with laboratory serial number written on it for an early morning specimen. Explain to the patient to rinse his/her mouth with plain water before bringing up the sputum.

Storage and transportation of specimens

If the specimen is collected in the field and cannot be immediately processed, it should be transported to the laboratory within 3-4 days of collection. The specimen should be collected in the containers meant for the purpose, lid tightly secured, properly labelled and kept away from the sun and heat. These can be placed in a special box which can withstand leakage of contents, shocks and other conditions incident to ordinary handling practices. These boxes should be kept in the cooler conditions and then transported to the laboratory.

Preparation of smear and Ziehl Neelsen staining (AFB staining)

- ➢ Select new unscratched slide and label the slide with Laboratory Serial number.
- ➢ Make a smear from yellow purulent portion of the sputum using a bamboo stick. A good smear is spread evenly, 2 cm x 3 cm in size and is neither too thick nor too thin. The optimum thickness of the smear can be assessed by placing the smear on a printed matter and the print should be readable through the smear.
- ➢ Let the smear air dry for 15-30 minutes.
- ➢ Fix the smear by passing the slide over the flame 3-5 times for 3-4 seconds each time.
- ➢ Stain the smear by Ziehl Neelsen method as described in Chapter 4.

Grading of microscopy smears

Record the results in laboratory form and laboratory register appropriately as per table given below:

Examination	Result	Grading	No. of fields to be examined
More than 10 AFB per oil immersion fields	Positive	3 +	20
1-10 AFB per oil immersion fields	Positive	2 +	50
10-99 AFB per 100 oil immersion fields	Positive	1 +	100
1-9 AFB per 100 oil immersion fields	Scanty	Record exact number seen	100
No AFB per 100 oil immersion fields	Negative	0	100

Store all positive and negative slides till instructed by supervisor to destroy them.

How to prevent false positive sputum results?

- ➢ Only use new, unscratched slides.
- ➢ Always use filtered carbol fuchsin.
- ➢ Do not allow the carbol fuchsin to dry during staining.
- ➢ Do not allow the carbol fuchsin to boil during staining.

- Decolorize adequately with sulphuric acid.
- Make sure there are no food particles or fibres in the sputum sample.
- Never allow the oil immersion applicator to touch a slide.
- Never allow the oil immersion lens to touch a slide.
- Label sputum containers, slides, and laboratory forms accurately.
- Record and report results accurately.

How to prevent false negative sputum results

- Make sure the sample contains sputum and not just saliva.
- Make sure there is enough sputum (at least 2 ml).
- Select thick, purulent portions to make the smear.
- Prepare smears correctly – not too thick, too thin or with too little material.
- Fix for the correct length of time, not too short or too long.
- Stain with carbol fuchsin for 5-7 minutes.
- Do not decolorize with sulphuric acid too intensively.
- Examine every smear for at least five minutes observing at least 100 fields before recording as negative.
- Label the sputum containers, slides and laboratory forms carefully.
- Record and report results accurately.

Culture for *M. tuberculosis*

Indications

Each and every case of suspected tuberculosis need not be cultured. The judicious use of culture limits its use to the followings:

- Diagnosis of smear negative pulmonary TB cases with strong clinical and radiological suspicion.
- Diagnosis of extrapulmonary TB.
- Follow up of a case to investigate for a drug resistant isolate.
- Diagnosis of childhood tuberculosis.

Procedure

In some of the intermediate laboratories, the culture for *M. tuberculosis* may be feasible – wherever indicated and possible. The procedure recommended is given below:

- In a small glass, sealable bottle, mix equal volumes of sputum and 4% sodium hydroxide.

- Shake well and incubate at room temperature (25-30°C) for 15-20 minutes with regular shaking every 5 minutes.
- Centrifuge at high speed (>13,000 g) for 8-10 mts.
- Discard the supernatant.
- Neutralize the sediment by adding drop by drop, 2 mol/litre HCl containing 20 ml of phenol red solution per litre until the mixture remains pink.
- Inoculate the neutralized deposit on to atleast 3 tubes of Lowenstein-Jensen (L-J) medium-one with glycerol and another with sodium pyruvate.
- Incubate the L-J tubes for 2-3 days at 35-37° C in a horizontal position with the caps loosened half a turn. Thereafter incubate at 37° C for 6 weeks and inspect for growth at weekly intervals. Initial incubation of the culture tubes in the presence of 5-10 percent CO_2 improves the growth of *M.tuberculosis*.
- Note the growth of bacteria on the surface during these weekly inspections, and if present stain by Ziehl-Neelsen method.
- If the isolate has the typical colonial appearance and the Z-N stained smear from a colony is also typical report the growth as *Mycobacterium* spp.
- Send the isolate to a reference laboratory for further characterisation and susceptibility testing.

> The growth of typical human strains is "rough, tough and buff" and can sometimes be seen after 2-3 weeks of incubation but seldom earlier.

Biosafety

- Treat all sputum samples as potentially infectious and use leak proof containers for collection and transportation.
- Use bacteriological safety hood while carrying out all procedures involving sputum.
- Dispose of the sputum cups by incineration, autoclaving or treating with 5% phenol or 2% freshly prepared hypochlorite solution, whichever is feasible.
- Take special precautions while transporting the cultures to the reference laboratories with emphasis on containers which don't get broken in the event of an accident.
- Wash hands with soap and water frequently especially after touching the sputum.
- Do not eat, drink or smoke in the laboratory area.
- Clean laboratory bench tops with a disinfectant (phenol or hypochlorite solution) at the end of each day.

Quality assurance

- All sputum smear positive slides and 10 percent of the randomly chosen negative slides be examined by the laboratory supervisor. There should be high degree of concordance between the results reported in the initial reading and that by the supervisor reading. If the persistence of false positive and false negative results is noticed, review all the analytical and para analytical factors.
- Quality assurance must be applied to safe laboratory arrangement, equipment, collection and transportation of specimen, reagents and methods and reporting of results. Use standard operating procedures, equipment should meet the manufacturer's claims and specifications and reagents should be of good quality. Analyze laboratory results on a weekly or monthly basis.Adequately trained and motivated staff is crucial to the generation of quality results.

Susceptibility to antituberculosis drugs

There is no need of undertaking susceptibility testing for anti-TB drugs as a routine. The resistance in tuberculosis has been found to be mainly due to noncompliance of treatment,rather than due to drug resistant bacilli. Moreover, the second line of drugs is very toxic, expensive and less effective than the first-line drugs. Anti-TB susceptibility testing is technically very demanding and should be done only in some selected reference laboratories.

Reporting of results

Sputum microscopy

- If all three specimens or two specimens show presence of AFBs: **Report positive.**
- If one of three specimens show AFB, correlate the findings clinically and radiologically.
- If none of the three specimens shows AFBs: **Report negative.**

Positive culture

Report positive when typical colonies are isolated and Ziehl Neelsen staining shows acid fast bacilli.

Negative culture

If no growth is obtained after 6-8 weeks of incubation report negative.

Referral

- As a quality assurance procedure
- For further characterization and storage of isolate
- Susceptibility testing in cases of treatment failure/relapses

Further reading

1. CH Collins, JM Grange, MD Yates: Organization and practice in tuberculosis bacteriology, Butterworths, 1985.
2. M Salfinger, GE Pfyffer. The new diagnostic mycobacteriology laboratory. Eur J Clin Microbiol Infect Dis 961-977, 1994.
3. AD Harris, D Maher, P Nunn Practical and affordable measures for the protection of health care workers for tuberculosis in low income countries. Bull WHO 75:477-489,1997.
4. DA Enarson, HL Reider, T Arnadottier, A Truebucq Tuberculosis guide for low income countries. IUTLD, Paris France, 1996.

18. Malaria

Malaria is a febrile illness caused by the presence of malaria parasites (*Plasmodium* sps.) in the human body. The parasite is found in the red blood cells of human beings. A person suffering from malaria will always get fever but all fevers are not due to malaria. The diagnosis of malaria is dependent upon the demonstration of malaria parasite in the peripheral blood film. The essential requirements for an accurate diagnosis of a case of malaria are:

- Making a proper blood smear from a patient having fever
- High quality staining of the smear
- Examination of the stained slide by a technician skilled in malaria microscopy

Preparation of blood smear

Malaria microscopy is the key to the diagnosis of malaria.

Equipment required

- Clean glass slides
- Pricking needle (Hagedorn triangular No 12)
- Spirit
- Cotton
- Slide box
- Lead Pencil
- Ball point pen
- Antimalarial drugs
- Record form or Register

Two types of smears are prepared from the peripheral blood – one thin smear and the other thick smear. Thick film examination is about 20 times more sensitive than thin film examination for parasite detection. Thin film examination is done for finding out the species of *Plasmodium*. Prepare the thin and thick blood smears in the following way:

- Hold the third finger of the left hand of the patient between your left thumb and finger at the first phalangeal joint.
- Wipe finger tip with swab dipped in savlon solution.
- Allow the finger tip to dry.
- Hold the pricking needle (Hagedorn triangular No.12) in right hand and prick the finger and allow blood drop to ooze out.
- Take a clean, dust free, grease free slide and take 3 drops of the blood 1 cm from the edge of the glass slide. Take another drop of blood one cm from the first drop of blood.

- ➢ Take another clean slide with smooth edges and use it as a spreader. Make thick smear by joining the 3 drops of blood and spreading it in an area of 10 mm diameter.
- ➢ Make thin smear by bringing in contact the spreader with the drop of blood at an angle of 30-45° from the horizontal and pushing the spreader steadily down the surface of the slide drawing the blood behind till the smear is formed.
- ➢ Allow it to air dry.
- ➢ Put the slide number on thin smear with lead pencil.

Qualities of good thick and thin smears are given below:

Thick smear

- ➢ It should be 10 mm away from the edge of the slide.
- ➢ It is round in shape with a diameter of 10 mm.
- ➢ Its thickness contains 10 layers of RBCs.
- ➢ 10-12 WBCs should be visible per oil immersion field of microscope.

Thin smear

- ➢ Uniformly spread over the slide.
- ➢ Thin enough so that a newsprint can be read through the smear.
- ➢ It is tongue shaped.
- ➢ Consists of a single layer of RBCs.
- ➢ Also used to label the identity of the patient.

Staining of blood films

Giemsa stain which is a mixture of eosin and methylene blue is widely used for staining the films. In some countries, JSB staining is used in the National Malaria Programme.

Giemsa staining

- ➢ Use slides which are well dried, peferably overnight.
- ➢ Fix thin film by dabbing it gently with a small piece of cotton dampened with methanol. Avoid methanol coming in contact with the thick film.
- ➢ Place the slides back to back in a staining trough making sure that all thick films are at one end of the trough.
- ➢ Prepare a 3% solution of Giemsa stain by adding 3 ml of Giemsa stock solution to 97 ml of buffered water.
- ➢ Pour the stain gently into the trough until the slides are totally covered. Do not pour the stain directly on to the thick films.
- ➢ Leave the slides in the stain for 30-45 minutes.
- ➢ Pour clean water gently into the trough to float off the scum on the surface of the stain. While pouring water, do not disturb the thick films.
- ➢ Pour off the remaining stain gently and rinse again in clean water for a few seconds. Pour off the water.
- ➢ Remove the slides one by one and place them, film side downwards, in a drying rack to drain and dry, making sure that the thick film does not touch the edge of the rack.

Examination of the stained slides

Since it takes almost 10 times as long to examine a thin film as to examine a thick film, in practice thick film is examined first. Thin film is examined only when the thick film gets autofixed or when it is necessary to confirm the identification of a species.

Examination of the thick film

- Using the 40x objective, select a part of the film that is well stained, free of staining debris and is well populated with WBCs.
- Place a drop of immersion oil on the thick film.
- Lower the 100x oil immersion objectives over the selected portion of the blood film, so that it touches the immersion oil.
- Confirm that the portion of the film is acceptable and examine the slide for 100 oil immersion fields by moving along the width of the slide.
- Examine atleast 100 good fields before a slide can be pronounced negative.
- Record your findings in an appropriate form.

Examination of thin film

- Place the slide on the mechanical stage and position the 100x oil immersion objective over the edge of the middle of the film.
- Place a drop of immersion oil on the edge of the middle of the film.
- Lower the oil immersion objective until it touches the immersion oil.
- Examine the blood film by moving along the edge of the thin film, then moving the slide inwards by one field, returning in a lateral movement and so on.
- Continue the examination, for atleast l00 fields to determine whether the blood film is positive or negative for malaria.

Recognition of the malaria parasite

There are four species of malaria parasite namely *Plasmodium vivax* (commonest), *P. falciparum* (second common spp.) *P.malariae* (less common) *and P.ovale* (rare). In all stages of development, the parasite will stain the same colour with Giemsa stain. These are:

- Chromatin which is part of the parasite nucleus is usually round in shape and stains a deep red.
- Cytoplasm occurs in a number of forms, from a ring shape to a totally irregular shape. It always stains blue, although the shade of the blue may vary between the parasite species.

The simplest guide to distinguishing between the four species of malaria is the effect the parasite has on infected red blood cells, whether or not it is enlarged and whether or not staining reveals Schuffner's dots or Maurer's dots. There are no RBCs in the stained thick blood film. The malaria parasites can be seen though they appear to be smaller than in thin blood films.

The essential morphological features distinguishing the four species of malaria parasite are given in Table 1. A pictorial description of the same has been given in Fig 1.

Table 1: **Differential characters of malaria parasites**

	P. falciparum	***P. vivax***	***P. malariae***	***P.ovale***
HOST CELL				
Size	Not enlarged	Enlarged	Not enlarged	Enlarged
Shape	Round and sometimes crenated	Round or oval (frequently bizarre form)	Round	Round or oval,often fimbriated
Colour	Normal but may become darkened	Normal but inclined to be pale	Normal	Normal
Stippling	Maurer's dots (large red spots)	Schuffner's dots (small red dots)	Ziemann's dots (few tiny dots not important for diagnosis)	James's dots (numerous small red dots)
Pigment	Usually black or very dark brown	Fine golden brown granules seen in cytoplasm	Black or brown coarse granules	Resemble more closely P. *malariae*
PARASITE				
General features	Small,compact dark, staining parasite. Multiple infections of single RBC	Large light staining parasite. Many trophozoites, may be amoeboid	Regular shape and moderate size. Strong tendency to form a band across the infected RBC	Regular shape. Size in between *P. vivax* and *P. malariae*
Common. Stages found in smear	Only rings and gametocytes	Trophozoites, Schizonts, Gametocytes	As in *P. vivax*	As in *P. vivax*
Ring stage	Delicate, small, 1.5 μm Double chromatin and multiple rings common. Accole, wing and marginal forms	Large 2.5 μm, usually single. Prominent thicker chromatin	Similar to *P. vivax* but thicker	Similar to *P. vivax*, more compact
Trophozoite	Compact, small, vacuole in-conspicous, seldom seen in smear	Large, irregular actively amoeboid prominent vacuole Chromatin as dots or threads	Characteristic band form, vacuole inconspicous	Compact coarse pigment, chromatin as large irregular clumps
Schizont	Small, compact seldom seen in blood smear	Large, filling the RBC, segmented, yellow brown pigment	Nearly fills RBC, like segmented, daisy head, pigment is dark brown	Fills three fourth of RBC, segmented Dark yellow brown pigment
Micro-gametocyte	Larger than RBC, kidney shaped with blunt round ends, cytoplasm reddish blue, fine granules scattered, many in number in smear	Fills enlarged RBC, round or oval, compact cytoplasm, pale blue, Abundant brown granules	Smaller than RBC, very few in PBF, round compact, cytoplasm pale blue. Pigment and chromatin as in *P. vivax*	Of the size of RBC round, compact very few in PBF, cytoplasm pale blue, chromatin and pigment as in *P. vivax*

Common defects in making blood films

- The blood films are not positioned properly on the slide making the examination difficult.
- Too thick a film makes it impossible to examine the smear after fixation.
- Too thin a film may not have adequate quantity of blood for detection of parasite.
- Blood film spread unevenly on a greasy slide makes examination difficult.
- Thin film too long, leaves less space for thick film.
- Autofixation of thick film occurs with passage of time or through exposure to heat making staining difficult.
- Thick film is allowed to dry unevenly.
- Wet slides are wrapped together and the slides stick to one another.

Keeping accurate records

It is important to keep complete record of the patient, so that you trace patients easily. Various parameters required are:

Patient's name, Age, Sex, Mailing address

Reporting of results

Laboratory Result

- Positive for malaria species, stage(s) seen.
- Negative for malaria.

Referral

- As a part of quality assurance programme.
- In malaria outbreak situations.
- In cases of detection of less common or rare species of *Plasmodium*.

Quality assurance

- To assess the quality of staining and blood film examination, a supervisor should re-examine a number of slides. Usually all positive slides and 10% of the randomly selected negative slides should be cross-checked.
- Monitor the quality of the preparation of thick and thin smears.
- Wash the slides clean and make these grease/dust free.
- Test the quality of the stains using known malaria positive slides at least once with every batch of the stain.

- Do not over stock the stains.
- Monitor the quality of the microscope periodically.

Biosafety

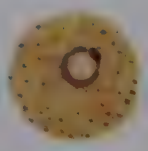
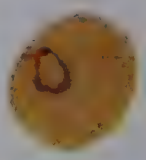
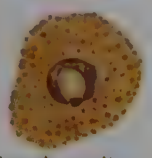
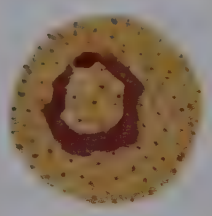
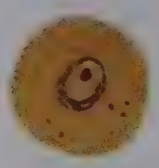
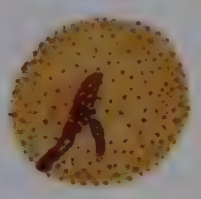
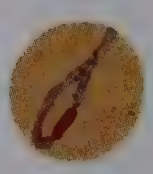
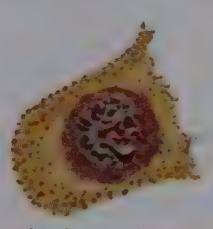
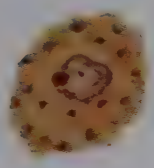
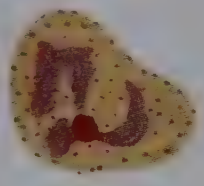
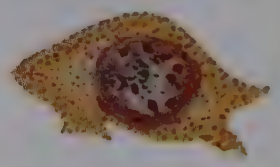
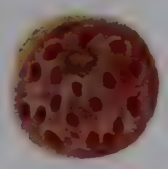
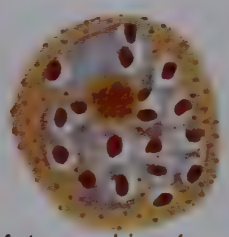
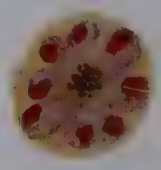
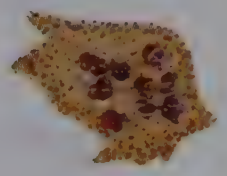
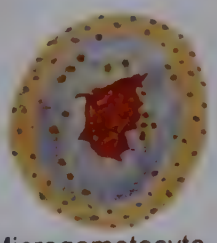
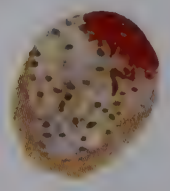
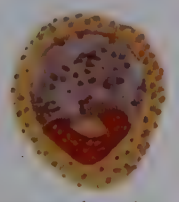
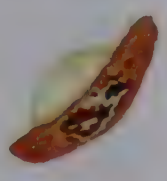
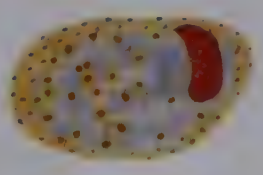
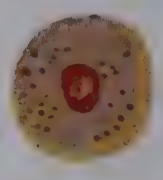
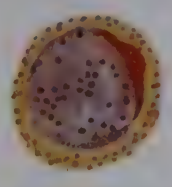
The collection and handling of blood samples presents a potential risk of transmission of diseases like malaria, viral hepatitis, HIV infection to another patient or health workers. Observe the following precautions.

- Wear protective gloves, as far as possible, when handling blood or taking blood samples.
- Cover any cuts or abrasions on your hands with adhesive dressing.
- Always wash your hands with soap and water after handling blood sample.
- If blood does get on to your skin, wipe it off quickly with cotton wool soaked with alcohol and wash the affected area with soap and water as soon as possible.
- Take care not to prick yourself accidentally.
- Never use disposable lancets more than once.

Further reading

1. Basic malaria microscopy: Learners guide, WHO, Geneva, 1991.
2. Bench aids for the diagnosis of malaria (8 plates) WHO, Geneva, 1988.
3. Instructions for malaria microscopists: Directorate of National Malaria Eradication Programme, DGHS, MOHFW, Govt of India, 1987.

Figure 1: *Morphological features of various plasmodia*

Plasmodium falciparum	***Plasmodium vivax***	***Plasmodium malariae***	***Plasmodium ovale***
Early trophozoite (Accole form)	 Early trophozoite (Ring form) with Schuffner's dots	 Early trophozoite (ring form)	 Early trophozoite (ring form)
Early trophozoite (double infection)	 Late trophozoite with Schuffner's dots	 Early trophozoite with central chromatin	 Developing schizont with enlarged red cell
Early trophozoite with Maurer's dots	Late trophozoite	 Early trophozoite form	 Developing schizont in RBC with hagged edges
 Late trophozoite with Maurer's dots	 Late trophozoite with amoeboid cytoplasm	Late trophozite band	 Developing schizont in an irregular red cell
 Mature schizont with Merozoites and clumped pigment	 Mature schizont with merozoites	 Mature schizont with merozoite forming a rosette	 Mature schizont with merozoites arranged irregularly
Macrogametocyte	 Microgametocyte	 Macrogametocyte	 Microgametocyte with irregular nucleus
 Microgametocyte	 Macrogametocyte	 Microgametocyte	 Macrogametocyte with compact nucleus

19. Urinary Tract Infection

Urinary Tract Infections (UTI) could be of the lower urinary tract encompassing the bladder and urethera or of the upper urinary tract infecting the ureters and kidneys. Because of shorter urethera, bacteria can reach the bladder more easily in females. All areas of the urinary tract above the urethera in healthy humans are sterile, hence urine is normally sterile.

UTI is among the most common reasons patients seek medical care. It is estimated that approximately 10% of humans will have UTI at sometime during their lives.

Causative organisms

E. coli is by far the commonest cause of uncomplicated community acquired urinary tract infections. Other members of Enterobacteriacae can also infect. The causative agents are listed in Table 1.

Table 1: ***Causative agents of UTI***

E. coli
Proteus mirabilis
S. aureus
S. saprophyticus
Group B streptococci
Enterococci

Complicated UTI which occurs in cathetrised patients or wiith obstruction is caused by:
E. coli
Klebsiella spp.
Enterobacter
Proteus spp.
Pseudomonas aeruginosa
S. aureus

Specimen collection

Prevention of contamination by normal vaginal, perineal and anterior uretheral flora is very vital. It is the responsibility of the laboratory to provide the patient with sterile, wide-mouthed glass or plastic, jars, beakers or other suitable receptacles which should have tight-fitting lids.

Though urine collected by suprapubic aspiration is the gold standard, it is not a practical method. Alternatively, mid-stream urine or a clean. catch urine is collected. Whenever possible, urine specimen should be collected in the morning, before the patient has voided urine.

The collection method is described as follows:

In men

- ➢ Instruct the patient to wash hands.
- ➢ Ask the patient to pull back the foreskin and pass a small amount of urine Holding back the fold of skin, instruct the patient to pass the remaining urine in a sterile container – this is mid-stream urine (MSU).
- ➢ Place the lid, secure tightly and rapidly transport to the laboratory.

  If the patient is bed-ridden, the nursing staff can help the patient in the above process.

The sample is best obtained, if the procedure is properly explained to the patient.

In women

- ➢ Instruct the woman to wash hands with soap and water before collection of specimen.
- ➢ Patient should undress in a suitable room, spread the labia and cleanse the vulva and labia thoroughly using warm soapy water.
- ➢ Rinse with warm water and dry.
- ➢ Ask the patient to pass urine, discarding the first part of the stream and collecting MSU in a sterile container.
- ➢ Transport the sample to the laboratory at the earliest after properly securing the lid.
- ➢ Bed- ridden patients can be assisted by the nursing staff.

In Infant and young children

- ➢ Ask the child to drink water or any other liquid.
- ➢ Clean the external genitalia and let the child be seated in the lap of the mother/nurse/attendant.
- ➢ Encourage the child to urinate and collect the same in sterile container.
- ➢ Cover the container tightly and rapidly transport to the laboratory for processing.

Collection of urine from catheters or bag should be avoided as this does not reflect the accurate picture. A reasonable alternative to MSU is the clean catch urine. After periuretheral cleaning the whole urine is collected into a sterile container and then an aliquot is sent for examination.

Specimen transport

Pending immediate processing of the sample, urine should be refrigerated. Bacterial counts at 4°C remains constant for 24 hours. Beyond 48 hours, even the refrigerated samples are not suitable.

Salient features of specimen collection

- ➢ Suprapublic aspiration of urine sample which is the best is not always possible.
- ➢ Mid Stream Urine and Clean Catch Urine are satisfactory alternatives.
- ➢ Process the sample at the earliest preferably within 4 hours.
- ➢ If immediate processing is not possible, refrigerate the sample at 4°C, upto a maximum of 48 hours.
- ➢ Catheter urine and bag urine are not ideal samples.

Screening procedures

Upto 80% of the urine specimen received in laboratory for culture may contain no etiological agent of infection or may contain only contaminants. There are various procedures tried to screen out such samples so that time, reagents and money of the laboratory is saved. Of these, a simple Gram stained smear of the urine has been found to be least expensive and probably the most sensitive and reliable screening method.

Gram stained smear of urine

- ➢ Place a drop of well mixed urine sample on a clean, grease-free slide.
- ➢ Air dry and heat fix.
- ➢ Perform Gram staining (Chapter 4).
- ➢ Examine under oil immersion (100x).
- ➢ Presence of atleast one organism per oil immersion field (examine at least 20 field) correlates with significant bacteriuria ($> 1 \times 10^5$/ml).

Culture

Urine is cultured in the following situations

- ➢ Sample screened by Gram stain and found to have significant bacteriuria.
- ➢ Follow up of patients on treatment.
- ➢ Urinary tract obstruction.
- ➢ Follow up after removal of indwelling catheter.
- ➢ Bacteremia of unknown origin.

Once it has been decided that urine sample is to be cultured, a measured amount is inoculated to each of the appropriate media. Usually a calibrated loop designed to deliver a known volume (say 0.0 I ml per loopful) of urine is used.

The method followed is as follows:

- ➢ Mix the urine sample thoroughly and remove the top of the container.
- ➢ Flame a calibrated wire inoculating loop and allow it to cool without touching any surface.
- ➢ Insert this sterile loop in the urine sample vertically and allow urine to adhere to the loop.
- ➢ Spread the loopful of urine over MacConkey agar plate using standard method.
- ➢ In the similar way, collect a second loopful specimen and inoculate a blood agar plate.
- ➢ Incubate the plates aerobically at 35-37°C for at least 24 hours.
- ➢ Count the colonies and count the CFU (colony forming unit) by multiplying with 100 (since 0.01 ml loop was used).

Guidelines for diagnosis of UTI are given as Chart 1.

Quality assurance

- ➢ Follow manufacturers' instructions for media.
- ➢ Follow IQC measures for laboratory equipment as recommended in Chapter 9.
- ➢ Periodically monitor the calibration of the inoculating wire loop.
- ➢ Monitor the quality of the urine sample by recording the storage temperature.
- ➢ Place the inoculating wire loop with handle down in a test tube rather than flat on the table to prevent change in the calibration.

Susceptibility to antimicrobial agents

Culture from patients seen in out-patient with uncomplicated UTI may not require a susceptibility test. Susceptibility tests are indicated on cultures obtained from patients who are hospitalised or have a history of recurring infection. In such cases follow the procedure as outlined in chapter 7 on antimicrobial susceptibility testing.

Reporting of results

- ➢ Report on number of WBC, RBC/ml of urine.
- ➢ Comment on the presence of epithelial cells, yeasts or parasites (e.g. *Trichomonas vaginalis).*

- If culture not indicated report "Culture not done because counts below signficant levels".
- Report on the type of organism isolated alongwith counts – significant or otherwise.
- Report absence of growth.
- Report susceptibility results as clinically indicated.

Referral

- Organisms with unusual resistance patterns.
- *Salmonella* spp. for further typing.

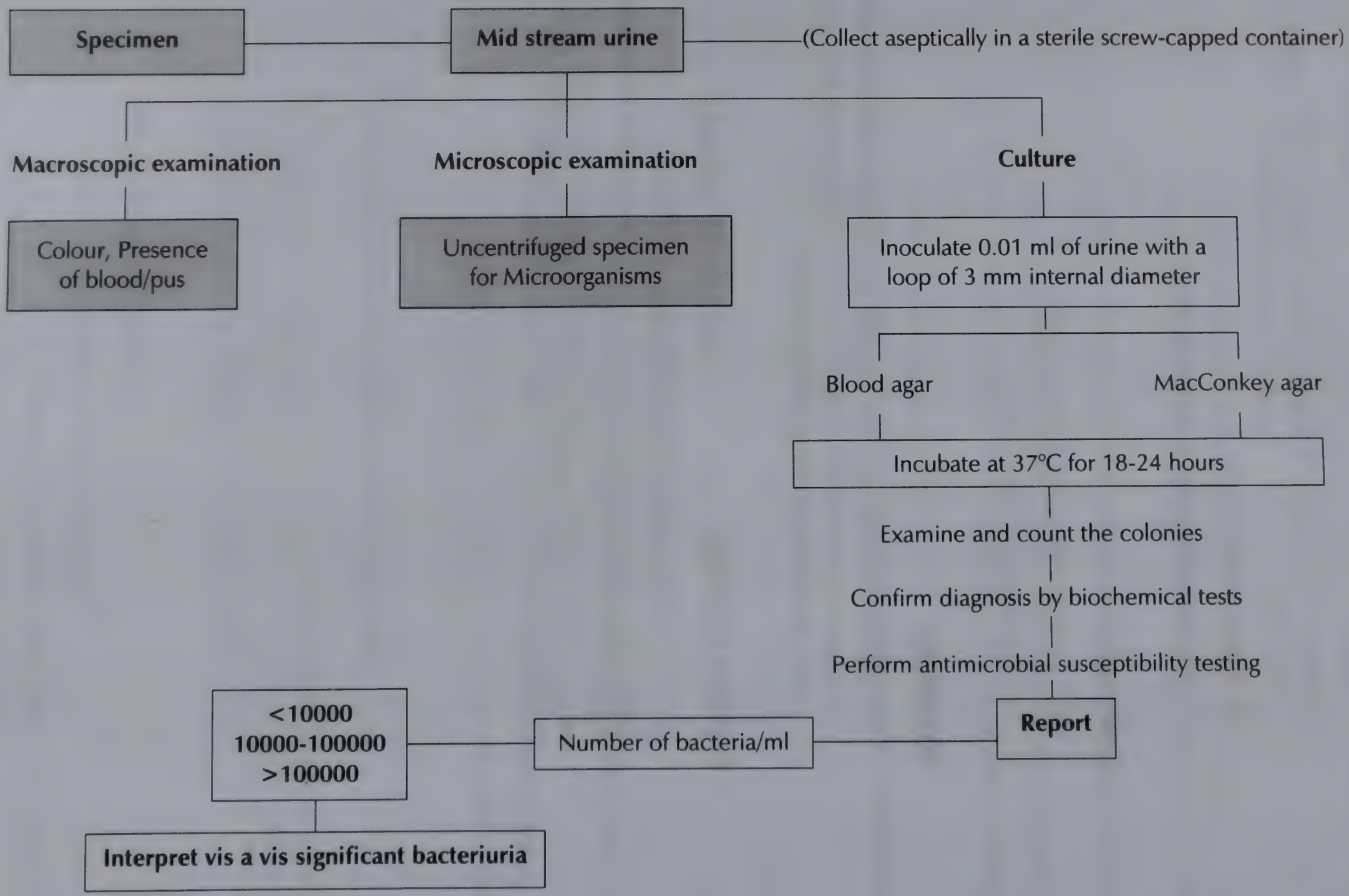
Biosafety

- Specimen transport and storage should be done in sterile leakproof containers.
- Practice routine laboratory biosafety measures particularly hand washing before and after collection/handling of clinical samples.

Further reading

1. Stevens M: Sceening urines for bacteriuria Med. Lab. Sc. 46: 194, 1989.
2. Stamm, W.E., Criteria for the diagnosis of urinary tract infection and for the assessment of therapeutic effectiveness. Infection 20 (Suppl 3): S-151.
3. Pezzlo M.: Urine culture Procedures Section 1.1.7 In lscnberg H.D. (Ed.) Clinical Microbiology Procedures Handbook Vol. I American Society for Nficrobiology, Washington DC, 1992.

Chart 1: Diagnosis of Urinary Tract Infection



20. Parasitological Examination of Faeces

The examination of faeces for parasitological diagnosis is done to detect:

- Adult worms
- Segments of tapeworms
- Ova and cysts
- Larvae
- Trophozoites
- Cellular exudates such as WBCs, RBCs, macrophages and Charcot-Leyden (CL) crystals

For this, the sample should be properly collected and preserved.

Collection of faecal sample

- Ask the patient to pass the stool sample directly into a waxed cardboard or a plastic cup with a tight fitting lid. Collection of sample in a match box or on plant leaves is not a satisfactory method.
- About 20-40 grams of well-formed stool or 5-6 table spoonfuls of watery stool will suffice for a routine examination.
- Ingestion of some medicines prior to collection of faecal sample may interfere with the detection of parasites. These include tetracyclines, sulfonamides, antiprotozoal agents, laxatives, antacids, castor oil, magnesium hydroxide, barium sulphate, bismuth kaolin compounds and hypertonic salts etc. These should not be taken 1-2 weeks before the examination of stool sample.
- All specimens must be properly labelled with patient's name, age, sex, and date of collection.
- The specimen must reach the laboratory within 30 minutes of passing of the stool, since amoebic trophozoites die and become unrecognizable after that.

Note

- Do not keep the specimen at warm temperatures. Try to keep it in cool, shady places.
- Prevent the drying of the specimen.
- Prevent contamination with urine or dirt particles.
- Multiple stool examinations are required before the presence of parasitic infections is ruled out.
- Stool should not be collected from bed-pans containing disinfectants.

Transportation of samples

If looking for trophozoites, stool specimen must be transported very rapidly to the laboratory to avoid disintegration of trophozoites. Stool samples should be examined within 30 minutes of collection of the specimen and not receipt of the specimen in the laboratory. Stool specimens should never be frozen and thawed or placed in an incubator because parasitic forms deteriorate very rapidly.

For permanent fixation of the stool specimen, 10% formol-saline (prepared by adding 100 ml formaldehyde to 900 ml of 0.85% sodium chloride) is a well known preservative. Polyvinyl alcohol (PVA) is a widely used preservative because the performance of concentration procedures and preparation of permanent stained smears are both possible with this.

Macroscopic examination

Various points to be noted are:

- ➢ Consistency: The consistency of the stool could be formed, soft, loose or watery. The cysts are found maximum in the formed stool while trophozoites are most abundant in watery stool.
- ➢ Presence of blood and mucus.
- ➢ Presence of round worms, thread worms or tapeworm proglottids.
- ➢ Colour and smell of the stool.

Microscopic examination (temporary wet mounts)

It is the simplest and easiest technique. A wet mount can be prepared directly from faecal material or from the concentrated specimens. The basic types of wet mounts that should be made from each sample include:

(a) *Saline wet mount:* It is used to detect worm eggs or larvae, protozoan trophozoites and cysts. In addition it can reveal the presence of RBCs and WBCs.

(b) *Iodine wet mount:* It is used to stain glycogen and nuclei of the cysts.

Procedure

- ➢ Place a drop of saline on left half of the slide and one drop of iodine on the right half.
- ➢ With an applicator stick pickup a small portion of the specimen (equivalent to the size of a match head) and mix with saline drop.
- ➢ Similarly pickup similar amount and mix with a drop of iodine.
- ➢ Put the cover slip separately on both and examine under the microscope.

- Ova, cysts, trophozoites and adult worms can be identified as per their characteristic features (Fig 1 and Table 1).
- Iodine wet mount is examined for amoebic and flagellar cysts.

Figure 1: *Morphological features of common parasites/eggs/ova/cysts*

a = *Entamoeba histolytica* trophozoite

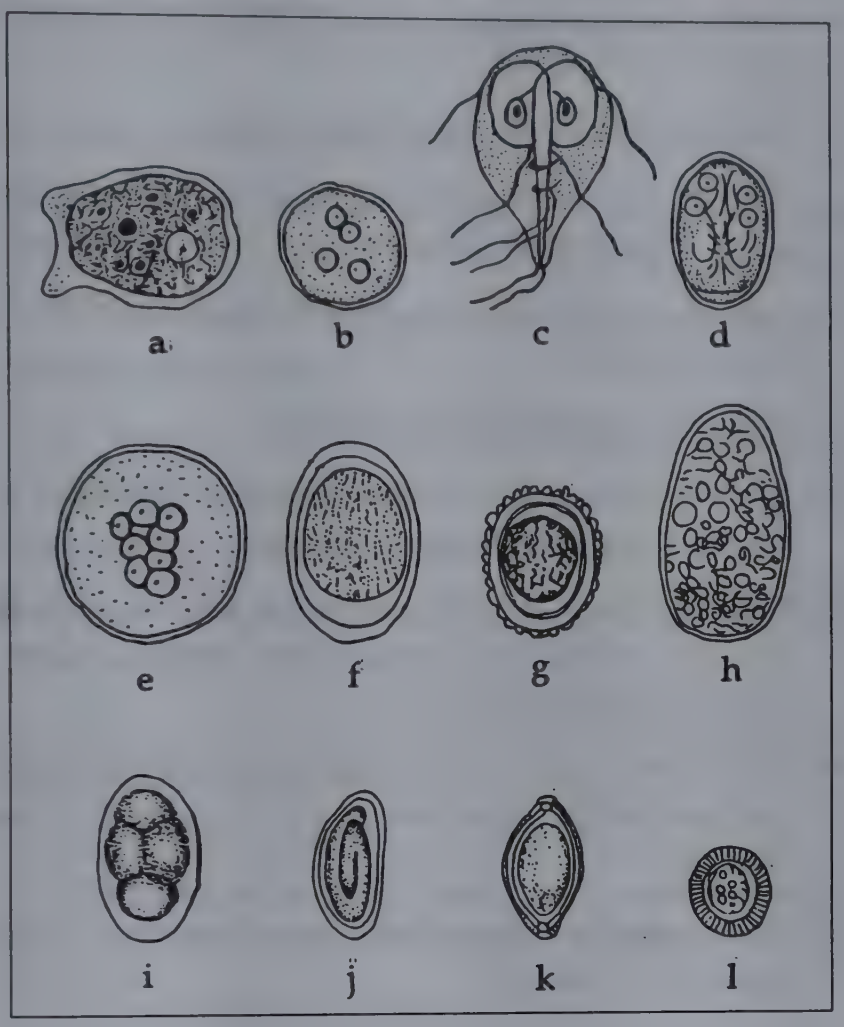
b = *Entamoeba histolytica* cyst

c = *Giardia lamblia* trophozoite

d = *Giardia lamblia* cyst

e = *Entamoeba coli* cyst

f = Decorticated egg of roundworm



g = Fertile egg of roundworm

h = Infertile egg of roundworm

i = Hookworm egg

j = Threadworm egg

k = Whipworm egg

l = Tapeworm egg

Concentration techniques

If the number of parasites in the stool specimens is low,examination of a direct wet mount may not detect them, hence the stool should be concentrated. Eggs, cysts and larvae are recovered after concentration procedures whereas trophozoites get destroyed during the procedure. This makes direct wet mount examination obligatory as the initial phase of microscopic examination.

The concentration procedures can be grouped under 2 categories:

(a) *Sedimentation procedures:* In which the eggs and cysts settle down at the bottom.

(b) *Flotation procedures:* In which the eggs and cysts float at the surface due to specific gravity gradient.

The basic disadvantage of sedimentation technique is that examination of the sediment is often difficult due to the presence of excessive faecal debris that may mask the presence of the parasites. The basic disadvantage of flotation technique is that not all eggs and cysts float in the flotation procedures.

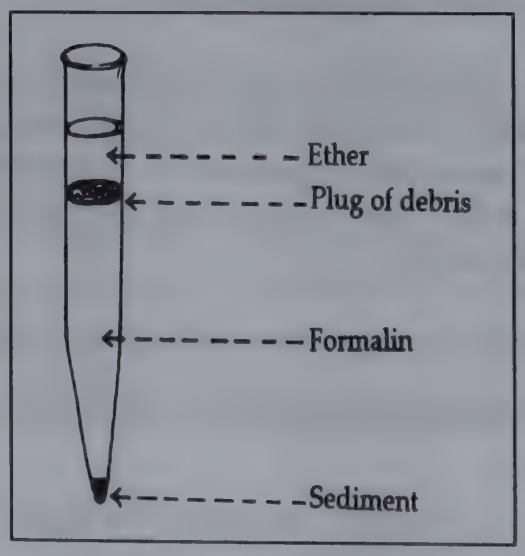
Two commonly used concentration techniques are formalin-ether and saturated salt solution technique.

Formal ether sedimentation technique

Procedure

- ➢ Transfer half teaspoonful of faeces in 10 ml of water in a glass container and mix thoroughly.
- ➢ Place 2 layers of gauze in a funnel and strain the contents into a 15 ml centrifuge tube.
- ➢ Centrifuge for 2 minutes at about 500 g.
- ➢ Discard the supernatant and resuspend the sediment in 10 ml of physiological saline. Centrifuge at 500 g and discard the supernatant.
- ➢ Resuspend the sediment in 7 ml of 10% formaldehyde (1 part of 40% formalin in 3 parts of saline).
- ➢ Add 3 ml of ether (or ethyl acetate).
- ➢ Close the tube with a stopper and shake vigorously to mix. Remove the stopper and centrifuge at 500g for 2 minutes.
- ➢ Rest the tube in a stand. Four layers now become visible the top layer consists of ether, second is a plug of debris, third is a clear layer of formalin and the fourth is the sediment (Fig 2).
- ➢ Detach the plug of debris from the side of the tube with the aid of a glass rod and pour off the liquid leaving a small amount of formalin for suspension of the sediment.
- ➢ With a pipette, remove the sediment and mix it with a drop of iodine. Examine under the microscope.

Figure 2: *Formal ether sedimentation technique*



Advantages

- ➢ Faecal odour is removed.
- ➢ The sensitivity of detecting the ova or cysts increases by 8-10 folds.
- ➢ The examination is easier than examining a direct wet smear.
- ➢ The size and shape of the parasitic structures is maintained.

- It is inexpensive, easy to perform and can be done at any level of health infrastructure.

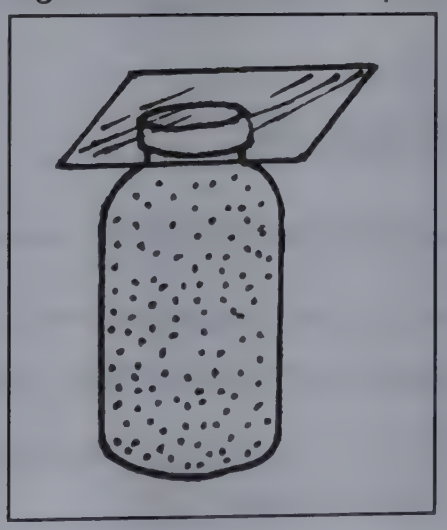
Disadvantages

- Faecal debris may mask the parasitic structure.
- Trophozoite forms are not detected in this method.

Saturated salt flotation technique

- Place about one millilitre of faeces in a container which is flat bottomed and has a diameter of less than 1½ inches and capacity of about 15-20 ml (Fig 3).
- Add a few drops of saturated salt solution (specific gravity 1.200) and stir it to make a fine emulsion.
- Add more salt solution so that the container is nearly full, stirring the solution throughout.
- Remove any coarse matter which floats up.
- Place the container on a level surface. Do the final filling by a dropper until a convex meniscus is formed.
- A glass slide 3″x 2″ is carefully laid on the top of the container so that the centre is in contact with the fluid.
- Preparation is allowed to stand for 20 minutes after which the glass slide is quickly lifted, turned over smoothly as to avoid spilling of the fluid and examined under the microscope after putting a coverslip.

Figure 3: *Flotation technique*



Disadvantages of flotation techniques

- Unfertilised eggs of *Ascaris lumbricoides*, eggs of *Taenia solium* and *Taenia saginata*, all trematodal eggs and larvae of *Strongyloides* do not float in the salt solution.
- Due to high specific gravity of the solution, protozoan cysts and thin walled nematode eggs will collapse and become distorted in appearance, if left for more than 20 minutes.

Biosafety

- Follow general laboratory principles for biosafety such as washing of hands, wearing of gloves, disinfecting work place and practising good personal hygiene measures.
- Handle the chemicals with care. Special precautions should be taken to store explosive chemicals (picric acid and phenol crystals) and flammable solvents such as acetone, ether, benzene, xylene etc.
- Equipment and glassware should be handled carefully to minimize risk of injury and aerosol production.
- Dispose off infectious material appropriately.

Disposal of morbid material

- After examination the stool specimen should either be incinerated or soaked in disinfectant solution and then buried in disposable specimen container.
- Used glass slides should be discarded in a pot containing 1% hypochlorite solution and cleaned if to be reused or buried if not to be used again.

Quality assurance

Attention be given to all pre-analytical, analytical and post-analytical factors affecting quality (Chapter 9).

Laboratories should volunteer to participate in external quality assessment schemes.

Reporting of results

The report should include positive/negative comments on the following:

- Adult worms/segments of worms/larvae.
- Cellular exudate such as RBCs, WBCs, Macrophages and CL crystals.
- Trophozoites (only if the specimen was fresh otherwise comment that specimen was not fit to comment on this).
- Ova and cysts.
- Any advise for further examination.

Referral

- As a part of any quality assurance programme.
- In case of unusual findings or an outbreak situation.

Table 1: **Salient features of common trophozoites, cysts and eggs of parasites**

Cyst/egg/trophozoite	Features
Entamoeba histolytica trophozoite	12-60 μ, asymmetric, purposeful directional motility, single spherical nucleus, single central karyosome, delicate and evenly distributed chromatin.
Entamoeba histolytica cyst	Spherical, 10-20 μ mature cyst has four nuclei with compact centrally located karyosome; chromatin is delicate. Some cysts may have chromatoid bars.
Giardia lamblia trophozoite	9-21x5-15 μ, pear shaped with tapering ends, actively motile like falling leaf, 2 centrally placed nuclei, uniform granular cytoplasm.
Giardia lamblia cyst	Oval, 8-12 μ long and 7-10 μ wide, nucleus has 4 karyosomes, tend to be eccenterically placed; clear space between cell wall and cytoplasm. Four median bodies are present.
Entamoeba coli cyst	10-35 μ, usually spherical, mature cyst may contain 8 or rarely 16 nuclei. Peripheral chromatin is coarse and granular; unevenly distributed in clumps; karyosome is usually eccentric. Chromatid bars not frequently seen.
Fertile egg of roundworms	60x45 μ, round or ovoid with thick shell; covered by a thick albuminous coat, inner cell in various stages of cleavage, brown in colour.
Decorticated egg of roundworm	Albuminous coat is lost. All other features are same as in fertile egg.
Infertile egg of roundworm	90x40 μ, elongated, shell is often thin, internal material is a mass of globules.
Hookworm egg	Oval, ellipsoid, 60x40 μ. Shell is thin walled, smooth and colourless. Internal cleavage is well developed at 4-8 cell stage which pulls away from the shell leaving an emplty space.
Threadworm egg	Planoconvex, elongate, asymmetric eggs, 55x26 μ, shell is thin and smooth. Fully developed larvae are seen in the eggs.
Whipworm egg	Elongate, barrel shaped with polar hyaline plug, 54-22 μ. Shell is yellow to brownish, plugs are colourless
Tapeworm egg	Spherical, 31-43 μ with thick shell with prominent radial striations. Embryonated oncosphere possessing 3 pairs of hooklets within the shell is diagnostic of the genus. Species identification on the basis of morphology is not possible.

Further reading

1. Ichhpujani RL, Bhatia Rajesh: Medical Parasitology. 2nd Ed Jaypee Bros, New Delhi 1998.
2. Elmer W Koneman et al: Colour Atlas and Textbook of Diagnostic Microbiology, 5th Ed, Lippencort, New York, 1071-1162, 1997.
3. Beaver PC, Jung RC, Cupp EW. Clinical Parasitology. 9th Ed, Philadelphia, Lea and Febiger, 1984.

21. Mycological Techniques

Skin and subcutaneous infections are frequently caused by various fungi. The clinical specimens required to be collected for mycological investigation of these infections usually are skin scrapings, hair and nail (clippings or scrapings). Care should be taken to collect adequate quantity of specimen. Sterilized instruments and containers should be used as far as possible to avoid contamination from external sources. Specimens collected must be brought to the laboratory and delivered for further analysis at the earliest, otherwise it will result in overgrowth with bacteria and other rapidly growing saprophytic fungi, which makes the recovery of the pathogens (which are relatively less rapidly growing) more difficult.

Collection and processing of samples

Most of the mycological diagnostic tests are highly specialized tests and services of a trained mycologist are absolutely essential for both performing the tests and interpreting them. However, intermediate laboratories can use simpler tests such as KOH mounts and India Ink preparations to detect fungal elements in the clinical specimens. If found positive the sample can be sent to reference laboratory for further processing or identification.

Mycotic infections present clinically as superficial mycoses, cutaneous mycoses, sub cutaneous mycoses and deep mycoses. For the sake of convenience, these are divided into superficial mycoses – including the infections of skin and its appendages and deep mycoses – including the sub-cutaneous and systemic mycoses which constitute the infections of deeper tissues and visceral organs.

Fungal infections are diagnosed by demonstration of the aetiological agent or its components in the clinical sample by:

(i) Direct microscopy by wet mounts and staining.

(ii) Isolation by culture and identification.

(iii) Histopathological diagnosis.

(iv) Immunological diagnosis which includes detection of antigen, antibody or a skin test.

All these methods are not feasible at the peripheral/intermediate laboratories. Intermediate laboratories can undertake direct microscopic examination and for the remaining, refer the sample to a reference laboratory.

Collection and processing of skin scrapings

- ➢ Thoroughly sponge the infected area with 70% ethanol to remove surface contaminants. If yeast infection is suspected replace ethanol with sterile saline solution or distilled water.
- ➢ Take the skin scrapings from the active border areas of lesions with the help of a sterile scalpel.
- ➢ Place the skin scrapings in sterile petri dishes or between two clean microscopic slides or in clean envelopes and transport to the laboratory.
- ➢ While collecting skin specimens it is important to note that any moist exudate present on the lesions should also be collected and examined. Exudative lesions are usually erythematous and painful and are generally caused by Candida.
- ➢ Place several scrapings in a drop of 10% KOH.
- ➢ Cover with cover slip.
- ➢ Pass several times over a flame to warm the mount gently.
- ➢ Examine the preparation under bright field microscope to detect fungal elements.
- ➢ If the slide is negative do not discard immediately but retain in a moist chamber and reexamine after few hours or a day.
- ➢ To culture, moisten the tip of a sterile, long handled inoculation needle by touching it to the surface of the agar medium (usually Sabourad dextrose agar-SDA).
- ➢ Incubate the culture at 27-30^{0}C and examine frequently for colonies.
- ➢ Identify the fungi recovered by referring it to nearest reference laboratory.

Collection and processing of hair

The technique for collecting hair specimen depends on the fungus involved. In the case of piedra, the hair bearing nodules may be clipped off with scissors. Dermatophytes first attack the roots of hair causing breaking of hair just above the roots. It is thus necessary to scrape the lesion with a sterile scalpel as well as to pull the stubs of broken hair with tweezers. Other details regarding collection and transportation remain same as for skin scrapings.

Collection and processing of nails

Scrapings of infected nails or clippings of nails are collected by shaving nails that have been cleaned with 70% ethanol. The scrapings are taken from the proximal to the distal end of the nail. The first 4-5 scrapings are discarded. The specimens are brought to laboratory and processed in the same way as skin scrapings are examined. For processing of nails, use 40% KOH instead of 10% KOH.

Collection and processing of other specimens

These may be collected for sending to the referral laboratories after initial direct microscopic examination at the local level.

CSF: The sample is collected aseptically as for other investigations. If there is delay in processing,store at room temperature. Do not refrigerate the sample. If culture is to be made, use as much quantity as is possible.

Blood: Collect about 5 ml of blood aseptically and incubate at 25-30°C till transported.

Bone Marrow: 2-3 ml of bone-marrow should be collected aseptically and transported to the laboratory at room temperature.

Tissue: Tissue should be placed in a sterile container with sterile saline without a preservative and transported to the laboratory. In case of delay refrigerate.

Urine: Collect preferably first early morning urine sample (though random samples are also acceptable) in sterile, screw capped containers. Place at 4°C in case of delay in transportation.

Exudates: The skin over pustular lesions should be disinfected and exudates aspirated using a sterile needle and syringe. Syringe can also be used as a transport container, if the needle is capped.

Presumptive identification based on direct microscopy

Various procedures which can be done for direct microscopic examination for detection of fungi in clinical specimen and are feasible at the level of the intermediate laboratories are as follows:

Acid fast staining	Used for detection of Mycobacteria and Nocardia.
Gram Stain	Most commonly used. Not only detects bacteria but can also detect most fungi.
India Ink preparation	Used for detection of *Cryptococcus neoformans*. When positive in CSF, is diagnostic of meningitis.
KOH preparation	Clears the specimen to make fungi more readily visible. Rapidly detects fungal elements.

Special fungal stains, are usually not available at intermediate laboratories and for them samples should be sent to a reference laboratory.

Some of the characteristic features of common fungi are listed hereunder:

Features seen on direct microscopic examination	Presumptive diagnosis
Small hyphae (2-3 μ), regular, some branching, sometimes with rectangular arthrospores found only in skin, nail scrappings and hair.	Dermatophyte group
Hyphae, pseudohyphae (distinct points of constriction) with budding yeast forms seen.	*Candida* spp.
Yeast forms, cells spherical and irregular in size (5-20 μ) with a thick capsule, with one or more buds attached by a narrow constriction.	*Cryptococcus neoformans*
Hyphae usually small (3-6 μ) and regular in size, dichotomously branching at 45° with distinct cross septa.	*Aspergillus* spp.
Hyphae irregular in size, ranging from 6-50 μ, ribbon like and are devoid of septa.	Phycomycetes *(Rhizopus, Mucor, Absidia).*
White or black soft or hard granules seen in exudate.	Suggestive of mycetoma

India ink mount

- Centrifuge the CSF sample at 1500-2000 g for 20 minutes.
- Mix a drop of this centrifuged deposit with a drop of India Ink on a clean microscope slide.
- Put a coverslip and examine the mount under the microscope for budding yeast forms.

Slide culture

The slide culture is used to study the undisturbed morphological details of fungi, particularly the relationship between reproductive structures and mycelia. By this technique the initial stages of sexual reproduction (formation and fusion of sex organs) may also be studied in some cases.

Procedure

- Prepare a moist chamber using a 9 cm diameter petri dish whose bottom is lined with two layers of filter/blotting paper disc, keep a V- or U-shaped glass rod at the bottom and sterilize.
- Pour about 15 ml of Sabouraud dextrose agar medium into a sterile petri dish; after sterilization mark the medium rapidly into 1 cm blocks using a flamed dissecting needle or a scalpel and a flamed glass rod.
- Scoop up an agar block from the plate and place it on a flame-sterilized and cooled microscope slide.
- Inoculate the four sides of the agar block with spores or mycelial fragments of the fungi to be studied.
- Place a flame sterilized cover-slip centrally upon the agar block.

- Transfer the slide culture (the microscope slide with the inoculated agar block) into the sterile, moist chamber already prepared, supporting the slide on V- or U-rod so that slide does not touch the bottom. Add a small quantity of sterile distilled water to keep the moist chamber humid; incubate at room temperature (25-28°C).
- Check the slide culture for growth and sporulation periodically. After sporulation has occurred two permanent stained slides may be prepared from each slide culture as follows:
  - Remove the cover slip from the agar block and apply a drop of 95% alcohol to the centre of the cover slip in order to wet the fungus; when the alcohol is nearly dry, add a drop of LCB mountant and mount the cover slip on a clean microscope slide; warm the slide to drive out the air bubbles, blot the excess LCB mountant and seal the edges of the cover slip with nail polish.
  - Remove the agar block from the slide and process the slide in the same way as described above; mount the fungus growth in LCB mountant using a clean cover slip; warm gently to drive out air bubbles, blot well and seal edges of the cover slip with nail polish.

Susceptibility testing to anti-fungal agents

Though a lot of technical advancement has been made in recent years for testing anti-fungal agents for susceptibility testing, yet a lot remains to be achieved to establish a technique which correlates *in vivo* susceptibility to *in vitro* results. Susceptibility is not tested even in most of the reference laboratories. The treatment is given as per the recommended drug therapy for a particular infection.

Biosafety

- Basic safety rules commonly followed in clinical microbiology laboratories generally suffice to protect laboratory workers.
- Examine all molds preferably in an enclosure such as a safety hood.
- Do not use procedures which encourage aerosol formation.
- Prevent accidental inoculation with sharp instruments.
- such as hypodermic needles, dissecting needles and scalpel blades.
- Avoid drinking, smoking, eating and application of cosmetics in the laboratory.
- Clean laboratory benches with a good disinfectant containing detergent.

Quality assurance

Quality control in a mycology laboratory does not in any way differ from the general principles described in Chapter 8. The quality checks should include all the analytic and para-analytical factors. Expertise of the technical personnel needs special emphasis for the diagnosis of fungal infections.

Reporting

(a) Report as direct smear examination suggestive of fungal infection. Write genus if features are suggestive.

(b) Report as direct smear does not show any evidence of fungal infection, if found negative.

(c) Report as sample sent to Reference laboratory, result awaited, in case of referral to the reference laboratory.

Referral

For identification/characterization of clinical samples from suspected cases of deep mycoses, facilities for which do not exist at the intermediate level laboratory.

Further reading

1. Emmons C.W., C.H., Baniford, J.P.Utz and K.J.Kwon-Chung. Medical Mycology. 3rd ed Lea & Febiger, Philadelphia, 1997.
2. McGinnis M.R. Laboratory handbook of medical mycology. Academic Press, New York, 1980.
3. Callaway C.J. and L.D. Haley. Laboratory methods in Medical mycology 4th ed. Centres for Disease Control, Atlanta, Ga. 1978.
4. Koneman, E.W., G.D. Roberts and S.F. Wright: Practical laboratory mycology 2nd Ed. The Williams and Wilkins Co. Baltimore 1978.

22. Bacteriological Examination of Water

Water, of adequate quantity and quality, is essential for healthy life. The associations between sanitation, water and health are well known. Many diseases are associated with contaminated water and water shortages. Without adequate water, people can neither wash themselves properly, nor their clothes, nor their cooking utensils and food cannot be prepared adequately or hygienically. These restrictions on cleanliness result in various parasitic, fungal and skin diseases, eye infections and diarrhoeal diseases.

Microbiological examination of water

Microbiological examination offers the most sensitive test for the detection of recent and potentially dangerous faecal pollution, thereby providing a hygienic assessment of water quality with high sensitivity and specificity. For this reason it is important to examine a drinking water source frequently by a simple test rather than infrequently by a more complicated test or series of tests. It is ideal to look for individual specific pathogen but it is not practical since they are few in number than the nonpathogenic organisms and methods to detect them are costly in time and money. Therefore indicators of human/animal pollution e.g. coliforms are used. Faecal streptococci are regularly present in the faeces in varying numbers but their number is fewer than *Esch. coli* and they probably die and disappear at the same rate. The presence of faecal streptococci alongwith coliforms in absence of *Esch. coli* is also confirmatory of faecal pollution.

Water sampling

Sources of water to be sampled

Water sources can be divided into three basic types for the purpose of sampling.

(a) Water from a tap or fixed hand pump

(b) Water from a reservoir (river, lake, tank)

(c) Water from a dug well

Sampling from a tap or pump outlet

- Remove any attachments from tap that may cause splashing.
- Wipe off the dirt from outside the tap.
- Turn on the tap at maximum flow rate and let the water flow for 1-2 minutes.

- ➢ Sterilize it for a minute with flame using gas burner, lighter or ignited cotton wool soaked in spirit.
- ➢ Open the tap and allow water to flow at medium rate for 1-2 minutes.
- ➢ Open the container for collecting the sample and fill the water by holding the bottle under the water jet. Leave a small airspace to facilitate shaking at the time of inoculation prior to analysis.
- ➢ Stopper the cap and label the container.

Sampling from reservoir

- ➢ Open the bottle under sterilized conditions.
- ➢ Fill it by holding it by the lower part, submerging it to a depth of about 20 cms, with the mouth facing slightly upwards. If there is a current, the bottle should face the current.
- ➢ Stopper the bottle and label it.

Sampling from a dug well

- ➢ Attach a stone of suitable size to the sampling bottle with a piece of string.
- ➢ Tie a 20 meter length of clean string on the bottle and to a stick.
- ➢ Open the bottle as described above and lower into the well.
- ➢ Immerse the bottle completely in water without touching the sides of the well and lower it down to the bottom of the well.
- ➢ Pull it out when the bottle is filled.
- ➢ Discard a little water to provide airspace.
- ➢ Stopper and label the bottle.

The sample is examined at the earliest preferably within one hour or should be quickly transported to the laboratory keeping in cool container away from sunlight. It should be positively examined within 6 hours of collection.

While sampling chlorinated water 0.5 ml of sodium thiosulphate solution (18 gm/L) should be added to sampling bottles to neutralize the residual chlorine present in water.

Methods of analysis

Two methods have been developed for the detection of indicator bacteria in water: multiple tube method and membrane filter method.

Multiple-tube method. In the multiple-tube method different amounts of water to be tested are added to tubes containing a suitable culture medium. The bacteria present in the water reproduce and produce acid with or without gas. From the number of tubes inoculated and the number with a positive reaction, the most probable number (MPN) of bacteria present in the original water sample can be determined statistically.

The multiple-tube method is applicable to all kinds of water : it can be used with clear, coloured, or turbid water containing sewage or sewage sludge, or mud and soil particles, provided that the bacteria are homogeneously distributed in the prepared test samples.

Method

Set up following volumes of different strengths of MacConkey broth in tubes/bottles each having an inverted Durham tube to detect the presence of gas and add specified volume of water as mentioned:

- One 50 ml of water to 50 ml of double strength medium.
- 5, 10 ml quantities each to 10 ml double strength medium.
- 5, 1 ml quantities each to 5 ml single strength medium.
- Incubate the tubes/bottles at 37°C for 18-24 hours.
- Observe change in colour and appearance of gas in Durham tubes in bottles.
- The media receiving one or more of the indicator bacteria show growth and a color change which is absent in those receiving an inoculum of water without indicator bacteria. Presence of both acid and gas indicates positive reaction whereas absence of either or both these features denotes a negative reaction.
- The presumptive positives are read and remaining negative bottles are reincubated for another 24 hours. Any further positives are added to the previous figures. The probable number of coliforms are read from the probability tables of McCrady (Table 1).
- From the number and distribution of positive and negative reactions, count of the **most probable number** (MPN) of indicator organisms in the sample may be estimated by reference to statistical tables. The test gives **presumptive coliform count** as the reaction observed may occasionally be due to the presence of some organisms other than coliforms.

Table 1: ***Most probable number of coliforms by McCrady's table***

No. of tubes giving positive reactions			
1x50 ml	5x10 ml	5x1 ml	MPN/100 ml
0	0	0	<1
0	0	1	1
0	0	2	2
0	1	0	1
0	1	1	2
0	1	2	3

No. of tubes giving positive reactions			
0	2	0	2
0	2	1	3
0	2	2	4
0	3	0	3
0	3	1	5
0	4	0	5
1	0	0	1
1	0	1	3
1	0	2	4
1	0	3	6
1	1	0	3
1	1	1	5
1	1	2	7
1	1	3	9
1	2	0	5
1	2	1	7
1	2	2	10
1	2	3	12
1	3	0	8
1	3	1	11
1	3	2	14
1	3	3	18
1	3	4	21
1	4	0	13
1	4	1	17
1	4	2	22
1	4	3	28
1	4	4	35

No. of tubes giving positive reactions			
1	4	5	43
1	5	0	24
1	5	1	35
1	5	2	54
1	5	3	92
1	5	4	161
1	5	5	>180

Eijkman test (differential coliform test or confirmed *E.coli* count)

Eijkman test is usually done to confirm that the coliform bacilli detected in the presumptive test are *E. coli.*, as some spore – bearing bacteria give false-positive reactions in the presumptive coliform test. After the presumptive test, subcultures are made from all tubes showing acid and gas to fresh tubes of single strength MacConkey medium which is brought to 37°C. These tubes are incubated at 44°C in thermostatically controlled water baths and examined after 24 hours. Those tubes showing acid and gas are that containing *E. coli,* the number is read from the McCrady table. *E.coli* can be confirmed by plating on solid media and testing for indole production and citrate utilization.

Frequency of examination

The daily sampling of water supplies serving a population for 10,000 or less is neither required nor practical. The emphasis should be on proper control of chlorination. The water supplies serving more than 100,000 population should be examined daily.

Standards

The classification of drinking water according to bacteriological tests is given below:

Class	Grade	Presumptive count (per 100 ml)	*E.coli* count (per 100 ml)
I	Excellent	0	0
II	Satisfactory	1-3	0
III	Suspicious	4-10	0
IV	Unsatisfactory	>10	0,1 or more

ST-100 PCT

Reporting

Mention the presumptive coliform count and *Escherichia coli* count per 100 ml of water

- Report: Fit/unfit for human consumption.
- Advise, if a repeat specimen is desired.

Referral

- In outbreaks of jaundice, cholera, typhoid fever etc.
- Monitoring of water quality.

Quality assurance

- Due emphasis should be given on proper collection and prompt transportation of the specimen.
- Refrigerate the water specimen for a maximum of 48 hours if not immediately processed.
- Ensure the reliability of media and instruments.
- Interpret the results properly.

Further reading

1. Report: Guidelines for drinking water supply. WHO Geneva, 1985.
2. Manual: Standards of quality for drinking water supply. Indian Council of Medical Research, New Delhi, Series No 44, 1975.
3. American Public Health Association, American Water Works Association and Water Pollution Control Federation: Standard methods for the examination of water and waste water 13th Ed, New York, 1971.

Section C
Guidelines on Collection and Transportation of Clinical Material to Referral Laboratories

23. Bacterial Food Poisoning

Bacterial food poisoning is a frequent public health problem especially in developing countries. This is mainly due to lack of proper hygiene while preparing the food items and lack of storage facilities for left over or partly used food. Bacterial food poisoning affects usually a group of people consuming common food contaminated with micro-organisms or their toxins during preparation or storage. Most of these cases are self limiting but some times persons may develop serious illness leading to considerable morbidity and mortality.

Causative agents

The common organisms responsible for food poisoning are:

- *Staphylococcus aureus*
- *Salmonella spp.*
- *Clostridium botulinum*
- *Vibrio parahaemolyticus*
- *Bacillus cereus*
- Other bacteria like *Campylobacter* and *Yersinia spp.* etc.
- Fungal toxins – *Aspergillus spp.*
- *Clostridium difficile* and *Clostridium perfringens*

Clinical microbiology laboratories are sometimes called upon to examine the foods during outbreaks of disease that might be food-borne. Microbiology laboratories can help in the following ways:

- Verifying the presumptive diagnosis.
- Help epidemiologists in identifying the vehicle of transmission.
- Determine the source and degree of infection.
- Trace the route of infection.
- Advise the epidemiologists on the type of specimen to be collected.

Collection of specimen

Depending upon the situation, the specimen may be collected from patients, controls, food handlers and food. The specimens should include the following:

- Serum, stool, vomitus and urine from patients and controls.
- Blood, spleen and liver tissue from fatal cases.
- Stool/rectal swabs, throat swabs and exudate/pus from lesions, if any, of food handlers.
- Sample of left over suspect food.
- Swabs of equipment/utensils in which food was processed before cooking.

The suspect foods are those that are implicated by the epidemiologist based on the attack rate. The general principles regarding collection of specimens are given hereunder:

- Collect specimens aseptically with a sterile implements and collect in sterile containers.
- Collect the specimens of the suspect food at the earliest.
- If the food article is solid cut it with a sterile knife and collect 100-200 grams of the sample from the centre.
- In case of liquids, first thoroughly shake the specimen to mix and then with the help of a sterile tube collect the specimen and shift into a sterile container.
- In case of raw meat or poultry, aseptically cut portions of meat/skin and put 100-200 grams in a sterile plastic jar and refrigerate the specimen.
- To collect the specimen from utensils and/or equipment in which food has been processed, moisten the swab with sterile 0.1% peptone water or buffered distilled water and swab the contact surfaces of the utensils. Then put the swab in an enrichment broth.
- Collect sample of water aseptically, used for cooking purposes in quantity ranging from 1-5 litres as feasible.
- All the specimens should be properly labelled and packed.

The following details should accompany the specimens:

- Place and address of location where outbreak has occurred.
- Presenting symptoms: nausea/vomiting/diarrhoea/fever/unconsciousness etc.
- Date of onset of symptoms.
- Date specimens collected.
- Method of collection and transportation.
- Epidemiological background and suspect organism.
- Condition of the food at the time of collection.

Transportation of specimen

The specimen of food collected for bacteriological analysis should be transported to the laboratory by the most rapid mode available. Perishable food should be kept at 2-8°C.

Hot food should be cooled rapidly by putting the containers under cold running water and then held at 0-4°C. Samples should be packed in such a way that there is no spillage during transportation. The receiving laboratory should be pre-informed about the method of transport and anticipated time of receipt in the laboratory.

Interpretation

- Pathogens are often easier to isolate from clinical specimens than from food. Correlate the isolate with epidemiological data before incriminating the causative agent.
- Organism is more likely to be the causative agent if the same organism (same serotype/phage type) is recovered both from suspect food and the clinical specimen taken from the patient.
- Food is confirmed as vehicle of toxic substance if organism or toxin (e.g. staphylococcal enterotoxin or botulinum toxin) are detected even in the absence of any clinical specimen.
- The source/mode of spread of the causative agent can often be ascertained if the agent is isolated from raw foods, food ingredients, equipment or food handlers or environment.
- Certain food items are more commonly associated with some pathogens and knowledge of this might help in confirmation of the diagnosis. Some examples of these are:

Milk and milk products:	*Staph.aureus*
Milk, milk products, egg, egg products	*Salmonella spp.*
Rice, cereals, soup	*Bacillus cereus*
Fish, shellfish	*Vibrio parahaemolyticus*
Raw fruits and vegetables	*Shigella* spp., parasites
Soft drinks, fruit juices	Chemicals(copper, zinc,lead)

Referral

- In case of outbreaks suspected due to organisms of food-borne origin.
- As part of medico-legal requirements.

Quality assurance

- General principles of quality assurance mainly focussing on the para analytical factors be adhered to. Special emphasis be given to proper collection and transportation of clinical specimens.
- The laboratory results should be interpreted with caution keeping in mind the clinical and epidemiological features.

Biosafety

General biosafety measures to be adopted in collecting and transporting the clinical specimens to the laboratory.

Further reading

1. Speck ML (Ed) Compendium of methods for the microbiological; examination of foods. 2ndEd, American Public Health Association, Washington, 1984.
2. Boyan FL Infections and intoxications due to other bacteria. In H Rieman and FL Bryan (Ed) Food Borne Infections and Intoxications 2nd Ed Academic Press Inc New York, 211-297, 1979.

24. Acquired Immunodeficiency Syndrome

Acquired Immunodeficiency Syndrome (AIDS) is a fatal disease state caused by Human Immunodeficiency Virus (HIV) 1 and 2, belonging to the Retroviridae family. The infection mainly occurs due to

- Heterosexual or homosexual promiscuity with infected partners.
- Blood transfusion with infected blood or blood products.
- Vertical transmission from an infected mother to the newborn.
- Intravenous drug abuse with unsterile syringes or shared syringes.

This is the result of complex reactions between the process of viral infection and its immune response and is characterized by depletion of cells of the immune system, especially T helper lymphocytes (CD_4 cells). Such a breakdown of the immune system leads to the development of opportunistic infections (bacterial, viral, fungal and parasitic) resulting in a spectrum of symptoms ranging from individuals in apparent good health to those with rapid disease progression and mortality from these infections or resultant malignancy.

The diagnosis of HIV infection is usually made on the basis of the detection of antibodies to HIV. Serological tests for detecting antibodies to HIV are generally classified as screening tests (sometimes referred to as initial tests) or confirmatory tests (sometimes referred to as supplemental tests). Initial tests provide the presumptive identification of antibody – positive specimens, and supplemental tests are used to confirm whether specimens found reactive with a particular screening test contain antibodies specific to HIV.

A variety of simple, instrument free initial tests are now available, including agglutination, immunochromatographic and dipstick tests. Specimens and reagents are often added by means of a dropper to the test device. A positive result is indicated by the appearance of a coloured dot or line, or shows an agglutination pattern. Most of these tests can be performed in less than 10 minutes, and are therefore called simple/rapid (S/R) assays.

UNAIDS and WHO recommend three testing strategies to maximize accuracy while minimizing cost. Which strategy is most appropriate will depend on the objective of the test and the prevalence of HIV in the population.

Strategy I (used for transfusion/transplant safety surveillance)

All serum/plasma is tested with one ELISA or simple/rapid assay. Serum that is reactive is considered HIV antibody positive. Serum that is non-reactive is considered HIV antibody negative.

When the objective is safeguarding the blood supply, the test selected for this strategy should preferably be a combined HIV-1/HIV-2 assay which is highly sensitive.

Strategy II (used for surveillance purpose)

All serum/plasma is first tested with one ELISA or simple/rapid assay. Any serum found reactive on the first assay is retested with a second ELISA or simple/rapid assay based on a different antigen preparation and/or different test principle (e.g., indirect versus competitive). Serum that is reactive on both tests is considered HIV antibody positive. Serum that is non-reactive on the first test is considered HIV antibody negative. Any serum that is reactive on the first test but non-reactive on the second test, should be retested with the two assays. Concordant results after repeat testing will indicate a positive or negative result. If the results of the two assays remain discordant the serum is considered indeterminate.

Strategy III (used for diagnosis of the disease in patients)

As in strategy II, all sera are first tested with one ELISA or simple/rapid assay, and any reactive samples are retested using a different assay. Serum that is non-reactive on the first test is considered HIV antibody negative. Serum that is reactive in the first test but non-reactive in the second assay, should be repeated with both tests. Strategy III, however, requires a third test if serum is found reactive on the second assay or is reactive on the repeated first assay. The three tests in this strategy should be based on different antigen preparations and/or different test principles. Serum reactive on all three tests is considered HIV antibody positive. Serum that remains discordant in the second assay, or is reactive in the first and second tests but non-reactive in the third test, is considered to be indeterminate. Serum that is reactive on the first assay on the second and third assays is considered indeterminate for individuals who may have been exposed to HIV in the last three months and negative for those who have not been exposed to any risk for HIV infection.

A schematic representation of the UNAIDS and WHO HIV testing strategies is given in Chart 1.

Collection of sample of blood

- Always wear gloves and use disposable syringes and needles.
- Collect 5 ml of blood aseptically from the patient.
- Allow it to clot.

- Serum is separated by centrifuging and serum transferred to clean screw-capped bottle/vial.
- Avoid addition of preservative since it inactivates the conjugates. If unavoidable, use thiomersal at a final concentration of 0.01%.
- Perform the ELISA spot test as per the manufacturer's instructions.

Transport

If facilities are not available to perform the test, transport the serum sample in tightly closed container, taking care to avoid breakage or spillage of the serum sample.

A proforma with details such as name, age, sex, risk factor, history of previous testing should accompany. Fix a biohazard symbol.

Storage

The sera samples are placed in leak proof plastic containers in the refrigerator at +4°C for storage. For long time storage deep freezing at -70°C is advised.

Quality control

1. Avoid HIV testing on lipaemic, haemolysed and contaminated sera, because the results are not reliable.
2. All specimen should be properly labelled.
3. Do not test body fluids (other than serum/plasma) with kits meant only for blood testing.
4. To validate the results with each test put appropriate controls.
5. Do not use date expired kits.
6. Interpret the data carefully using the test kit inserts.
7. Keep in mind that false positive results can be obtained in chronic alcoholics, parentral drug abusers, multiple pregnancies and multiple blood transfusions.
8. Record the results with utmost care.
9. The laboratory should participate in an External Quality Assessment Scheme.

Referral

- For confirmation of diagnosis.
- As a part of quality assurance programme.
- In the event of unusual observations.

Biosafety

Guidelines for good laboratory biosafety practices as outlined in Chapter 8 should be strictly followed.

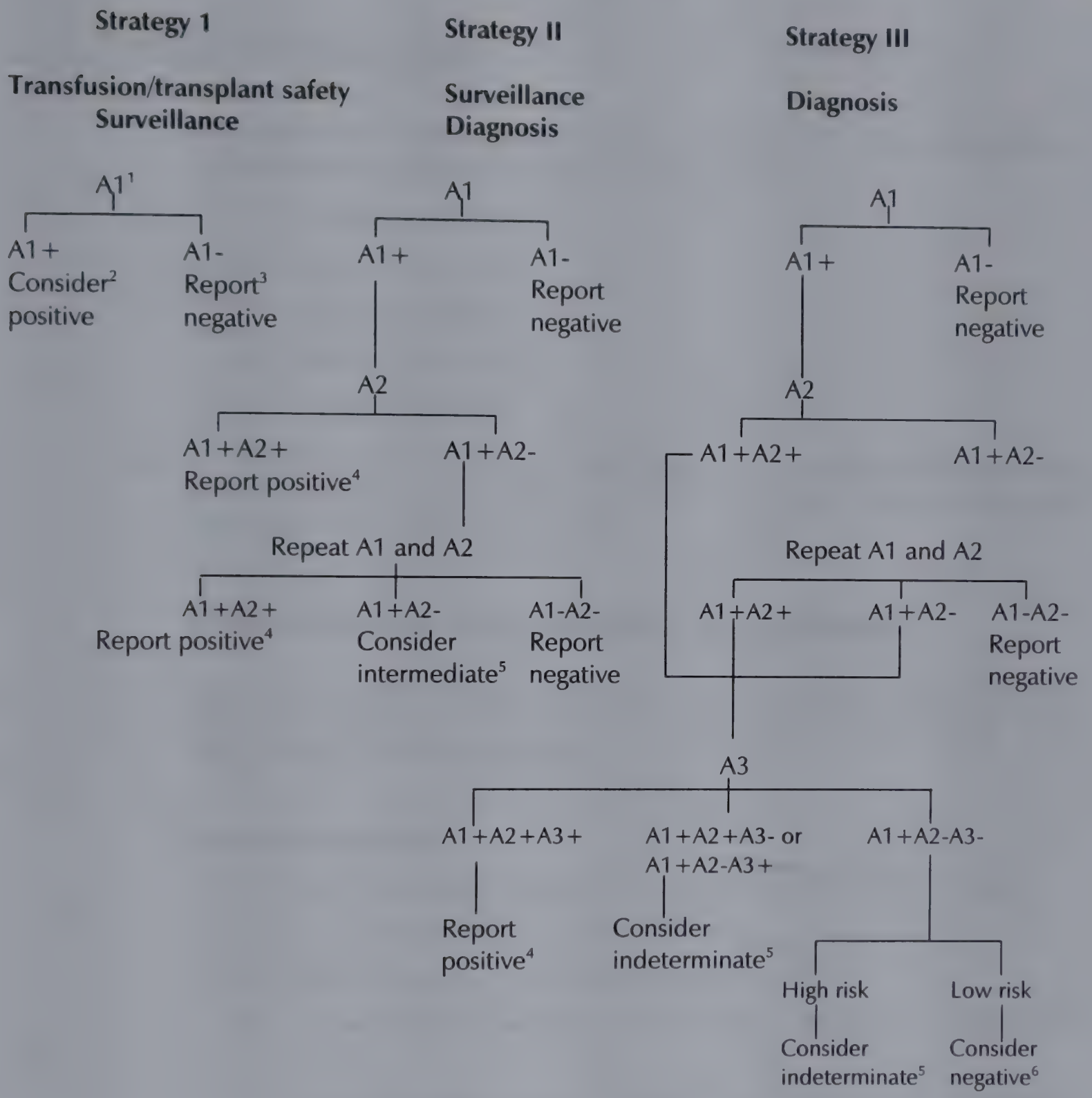
Reporting of results

This should be done as per the chart 1.

Further reading

1. Revised Recommendations for the selection and use of HIV antibody tests. WHO/UNAIDS, 1998.
2. Human Immunodeficiency Virus: Laboratory Training Manual. ASTPHLD, Connecticut, Ave, Washington, D.C. 1994.
3. Operational characteristics of Commercially available assays to determine Antibodies to HIV-1 and/or HIV-2 in Human sera Document No.WHO/BLS/98.1 UNAIDS/98.14.

Chart 1: Schematic representation of the UNAIDS and WHO HIV testing strategies



[1]Assay A1, A2 and A3 represent three different assays

[2] Such a result is not adequate for diagnostic purposes: use strategy II or III. Whatever the diagnosis, donations which were earlier reactive should not be used for transfusion or transplant

[3]Report: result may be reported

[4]For newly diagnosed individuals a positive result should be confirmed on a second specimen

[5]Testing should be repeated on a second specimen taken after 14 days

[6]Result is considered negative in the absence of any risk of HIV infection

25. Viral Hepatitis

Viral hepatitis is a systemic disease primarly involving the liver. The viruses which are recognised until now and for which tests are available are:

- Hepatitis A virus (HAV)
- Hepatitis B virus (HBV)
- Hepatitis C virus (HCV)
- Hepatitis D virus (HDV)
- Hepatitis E virus (HEV)

Of these HAV and HEV are transmitted faeco-orally while the remaining three viruses are blood borne.Large scale outbreaks of water-borne jaundice are usually due to hepatitis E virus and rarely caused by hepatitis A virus.

Collection and transportation of specimens

The role to be played by most of the peripheral and intermediate laboratories in the diagnosis of viral hepatitis may only be that of collection of blood sample and its transportation to a reference laboratory.

Collection of blood

- Collect blood sample aseptically using disposable/sterile needle and syringe.
- Draw a minimum of 3-5 ml of venous blood.
- Collect the blood in a sterile, dry and labelled vial.
- Separate the serum and store it in a refrigerator, if facilities for deep freezer do not exist.
- In outbreak situation, specimen should be collected from patients with disease of different duration.

At most of the intermediate laboratories, HBsAg detection can be undertaken by RPHA and latex agglutination tests. However, these tests are not very dependable. As far as possible, blood specimens should be screened by ELISA.

Latex agglutination test for detection of HBsAg

Commercial kits for this test are available. These contain the following reagents and accessories:

Reagent 1	HBsAg latex reagent	1 vial
Reagent 2	Positive control serum	1 vial
Reagent 3	Negative control serum	1 vial
Accessories	Disposable plastic slides Disposable applicator sticks Disposable plastic droppers Rubber teats	

All the reagents are stable and active, till the expiry date mentioned, provided they are stored in a refrigerator at -2 to 8°C.Do not freeze the reagents.

Latex agglutination test is performed on serum harvested from the patient/donor's blood. Do not inactivate the test or the control serum samples.

Test procedure

- ➢ Allow the reagents to attain room temperature.
- ➢ Shake the vials gently to make sure that the latex reagent is completely in suspension.
- ➢ Place one drop (50 µL) of undiluted serum in one of the circles on the slide. Fill more circles if more than one test serum samples are to be tested. Use separate droppers for each specimen.
- ➢ Add one drop (50 µL) of latex reagent onto each specimen drop in circles, using a disposable dropper.
- ➢ Mix the contents of each circle using separate disposable applicator sticks for each circle, and spread the mixture uniformly over the entire area of the circle.
- ➢ Rock the slide gently, to and fro, for 5 minutes, and watch for agglutination.

Interpretation

No agglutination	HBsAg negative
Visible agglutination within 5 minutes	HBsAg positive

However, the results must be interpreted with caution since latex agglutination tests fail to detect about 10% of HBsAg positive blood donations.

The specific aetiological diagnosis of viral hepatitis is cumbersome and is possible in few selected laboratories where infrastructure and technology are available to detect a variety of antigens and antibodies that are generated by different viruses. Various markers of viral hepatitis and their importance is:

HAV : IgM HAV indicates acute infection with HAV
IgG HAV indicates past infection with HAV and also immunity to HAV

HBV : HBsAg indicates infectivity
IgM HBc indicates acute infection with HBV
IgGHBc indicates past exposure to HBV
Anti HBs indicates protection against HBV
HBeAg indicates presence of actively replicating virus and superinfectivity.

(A blood sample having HBsAg indicates that the specimen is infectious and if it contains HBeAg it will be considered as highly infectious. HBsAg positive blood samples are not transfused and hence the test to detect this antigen is a screening test to ensure safety of blood before transfusion)

HCV : IgG HCV indicates exposure to HCV and also denotes that the blood sample is infectious

HDV : It is a defective virus which requires presence of HBV for its survival
IgM HDV indicates acute delta virus infection

HEV : IgG HEV indicates past exposure to HEV
IgM HEV indicates recent exposure to HEV

The testing of these markers is required for:

- Diagnosis
- Prognosis
- Safety in blood transfusion
- Assessment of exposure
- Detection of carrier state
- Seroconversion following immunization

Reporting of results

- Report the results of markers tested alongwith interpretation mentioning the test method used.
- Report negative result for the marker tested.
- Report should specify if any other tests are required.

Quality assurance

- Collect 3-5 ml blood aseptically, prevent haemolysis, label properly and transport to the laboratory under recommended conditions.

- All the factors related to analytical components such as use of SOPM, good quality reagents, instruments and training and skills of laboratory technical staff are given importance.
- Use positive and negative controls on tests wherever indicated.
- The results should be interpreted and communicated to the user. Mere writing the presence or absence of various markers is not recommended.
- Along with the laboratory results, always mention the type of tests performed while reporting.

Biosafety

Viral hepatitis spreads by faeco-oral (A & E) and parenteral route (B,C and D) and hence the biosafety measures are to be focussed on both the aspects. Hepatitis B has emerged as the commonest laboratory acquired infection. The biosafety steps recommended are as follows:

- Biosafety level 2 (BSL-2) practices and facilities are recommended while handling clinical material from hepatitis cases.
- All personnel concerned with handling the clinical material should be screened for HBsAg and if negative must be vaccinated against hepatitis B.
- Mouth pipetting, smoking, eating or drinking in the laboratory should be strictly forbidden.
- Needles/syringes used in the laboratory should be autoclaved before being discarded.
- Work areas should be decontaminated with 0.5% sodium hypochlorite prepared fresh each month.
- Disposable gloves are worn when working with known infectious materials.

Referral

- In outbreak situations
- For confirmation of diagnosis
- As a part of quality assurance programme

Further reading

1. Guidelines for preventing HIV, HBV and other infections in the health care setting. WHO-SEARO, New Delhi 1996.
2. Lennette H Edwin, Albert Balows, WJ Hansler and HJ Shadomy Hepatitis viruses In Manual of Clinical Microbiology 4th Ed, ASM, Washington, 813-836, 1985.

26. Poliomyelitis

Poliomyelitis is caused by poliovirus which is a RNA virus with three serotypes 1,2 and 3. The disease is approaching eradication phase which puts extra importance on the role of laboratories in peripheral and intermediate level since every case of poliomyelitis shall now require laboratory confirmation of diagnosis.The monitoring of potency of oral polio vaccine (OPV) is also now routed through peripheral laboratories. For both these activities the tests are to be performed only in designated reference laboratories. The methods of collection and transportation of specimens are important from the peripheral and intermediate laboratory point of view.

Collection of stool specimen from a case with acute flaccid paralysis (poliomyelitis)

The quality of diagnosis depends upon the correct timing and collection of clinical specimens and their proper transport to the laboratory. This requires close cooperation between virologist, epidemiologist and clinician. Because polioviruses multiply in the intestinal tract for several weeks after infection, specimen of faeces is most suitable for virus isolation.

- ➢ Collect specimen for virus isolation as early in course of illness as possible, but definitely within 14 days of onset of paralysis.
- ➢ Collect two specimens at an interval of 24-48 hours since virus excretion may be intermittent.
- ➢ Collect about 8 gm (about the size of the tip of thumb) in a clean, leak proof screw cap container, preferably in transport medium like Minimum Essential Medium or Eagles medium.
- ➢ Seal the container with tape.

Specimen taken from a suspected case of poliomyelitis is identified nationally and internationally by a standard case investigation number known as EPID number. This is used as common reference by case investigator and laboratories. EPID number is allocated by the senior epidemiologist.

A laboratory request form duly filled should accompany every specimen. One such form is included in each diagnostic kit. The format recommended by Global Programme for Vaccines and Immunization for this purpose is given as Annexure.

Transportation of specimen

- ➢ After collection, immediately place the specimen in a refrigerator at 2-8°C or in a cold box with frozen ice-packs.
- ➢ Transport the specimen at this temperature and it must reach the laboratory within 72 hours of collection.
- ➢ Disinfect or destroy the material that are used to transport the specimen.
- ➢ The set of specimens relating to a single case should be placed in a single plastic bag just large enough to hold both the containers.

Collection of other specimens

Necropsy specimens from the central nervous system can be collected as soon as after death as possible and transported under cold conditions. Throat swabs and blood specimen are not ideal specimens and hence not recommended.

Collection of OPV sample for potency testing

OPV is the most thermolabile vaccine of the 6 vaccines currently in use in EPI. To assess the quality of cold chain, OPV is taken as an indicator vaccine. A reverse cold chain is applied wherein OPV specimen is collected from the delivery point and sent to the reference laboratory for testing. The method of collection is as follows:

- ➢ Select a used or unused vial of OPV. The used vial must have at least 2 ml of vaccine.
- ➢ Affix an adhesive label; on the bottom of the vial stating the name of the health facility and date of collection. Alternatively, put only serial number on the label and refer other details in accompanying proforma.
- ➢ Ensure that the manufacturer's label is intact.
- ➢ Place the labelled vial of OPV in a polythene bag and affix second label indicating the name of health facility on the polythene bag.
- ➢ Roll the polythene bag around the sample and secure it by a rubber band.
- ➢ Fill up the details as required by the laboratory where sample is to be sent.
- ➢ Place the polythene bag containing the sample in a thermocol box/thermos containing enough wet ice.
- ➢ Drain the water from flask/thermocol box from time to time.
- ➢ Replenish fresh wet ice if required during the transportation to the laboratory.

Reporting of results

The reference laboratory gives results as follows:

For OPV

Satisfactory : when the titre of all three types is within acceptable limits.

Unsatisfactory: when the titre of even one type is not within acceptable limits.

For virus isolation

Poliovirus isolated	Yes/No
If yes	Type 1/2/3/mixture
Wild virus/vaccine strain	
Nonpoliovirus isolated	Yes/No

Referral

Peripheral laboratories refer the specimens for the following reasons:

- Potency testing of OPV for monitoring of cold chain.
- AFP surveillance.

Quality assurance

The laboratory must strictly implement internal quality control methods covering both analytical and paraanalytical components.

The laboratory must participate in an external quality assessment scheme. Laboratories are accreditated as members of WHO laboratry network for polio eradication.

Biosafety

After the vaccination against poliomyelitis became available and biosafety measures implemented there are no laboratory associated infections reported. However, recent evidence indicates that potential exists for the transmission of wild poliovirus from the laboratory to the community. Reintroduction of wild poliovirus from the laboratory to the unimmunized community remains a serious risk.

Following biosafety measures are recommended:

- The laboratory work should be undertaken in biosafety level-2 laboratory only.
- Good microbiological techniques are practised.
- Access to the laboratory is limited and the persons entering the laboratory should be fully immunized against poliomyelitis.

- All potentially infectious materials should be autoclaved on site and subsequently incinerated.
- the materials likely to contain wild polioviruses should be transported in triple packaging system.
- Use of wild polioviruses be discontinued where attenuated vaccine poliovirus or inactivated antigens could be satisfactory alternative.

Further reading

1. Manual for virological investigation of polio WHO/EPI/GEN/97.01 Geneva 1997.
2. Madeley CR: Guide to collection and transport of virological specimens WHO, Geneva 1997.
3. World Health Organization. Lboratory biosafety manual, Geneva, 1995.

Annexure

LABORATORY REQUEST FORM

Country

EPID No

Patient's name

M F

Address

Village/town/city

Commune

District

State/Province/Governorate

Date of birth of patient

DD/MM/YY

If date of birth is not known, give age in months

Date of onset

Date of first stool specimen collected

Date second stool specimen collected

Date stool specimen sent

Date of last dose of OPV

Preliminary clinical diagnosis

Clinical diagnosis in hospital

Name of person to whom laboratory results should be sent

Complete address

Telephone/Fax No.

For use in the receiving laboratory

Date specimen received in the laboratory DD/MM/YY

Name of person receiving specimen in lab

Was specimen in good condition YES/NO

(Adequate volume, no leakage, no desiccation and temperature indicator of presence of ice indicating reverse cold chain was maintained)

27. Dengue Fever and Dengue Haemorrhagic Fever

Dengue fever (DF) is an acute febrile viral disease presenting with headache, bone or joint and muscular pains, rash and leukopenia. Dengue haemorrhagic fever (DHF) is characterized by high fever, haemorrhagic phenomenon often with hepatomegaly and in severe cases signs of circulatory failure. Such patients may develop hypovolaemic shock resulting from plasma leakage which is then called as dengue shock syndrome (DSS).

Epidemics of dengue fever or dengue-like disease are reported from many countries of the South-East Asia Region. The peripheral/intermediate laboratories play a vital role in confirming the diagnosis of the disease by performing some of the tests at their own level and by sending the clinical specimens to the reference laboratories for confirmation of the diagnosis.

Laboratory diagnosis

Laboratory diagnosis is based upon two main methods.

(1) Detection of virus

This is done by isolation of virus or demonstration of antigen. These are definitive diagnostic tests. However, only a few laboratories perform these tests. This requires specific infrastructure and skilled manpower.

(2) Serodiagnosis

This is performed by detection of dengue specific antibodies. The serological tests are simple, rapid and most laboratories can perform these. However, accurate diagnosis cannot be made with these as cross reactions with other flaviviruses occur.

Collection and transportation of specimens for serodiagnosis

Usually three blood specimens are collected which help in proper interpretation of results

S1	:	At admission
S2	:	10-14 days after S1
S3	:	14-21 days after onset of disease

Blood specimens should be collected aseptically in tubes/vials. Filter paper method can also be used for collection and transportation of the specimen (see Chapter 2).

Transportation

If delay is anticipated, separate the serum and transport it to the laboratory on wet-ice at the earliest. Do not send frozen whole blood. When the specimen is collected on filter paper the same may be sent wrapped in a polythene bag, at room temperature, preventing it from exposure to moisture during transportation.

Specimen suitable for culture

In outbreak situations, the intermediate laboratories may be required to collect and transport specimens to referral laboratories for culture or demonstration of viral antigen. The important points in this exercise are:

- Collection of specimen early in the course of disease usually within 5 days of onset of fever.
- Prompt delivery to the laboratory.
- Dengue virus is heat labile, specimens awaiting transportation should be kept in refrigerator or packed in wet ice.

The specimens used for isolation of the virus from the suspected patient are:

- Serum/plasma
- Washed leucocytes
- Cerebrospinal fluid

From autopsy

- Homogenized liver, lung, spleen, lymph node, CSF, pleural fluid.
- The virus can also be isolated from vector mosquito.

Information accompanying the specimen

Following information must accompany the specimens:

- Name, age, sex of the patient
- Name of father/mother
- Complete address
- Name of the hospital sending the specimen
- Registration number of the patient
- Date of onset of illness
- Date of hospitalization

- Date of collection of specimen and number of specimen
- Brief clinical history
- Provisional diagnosis

Recognizing cases of DF/DHF/DSS

An outbreak of DF/DHF in the community should be suspected when:

- Children are suffering from undiagnosed fever of 2-7 days duration.
- Patients have petechiae, bleeding from nose or gums, melaena or haematemesis.
- Patients remain ill despite a drop in temperature and develop signs of shock.
- Unexplained death due to shock, with or without haemorrhage.

Rapid Immunochromatographic test for detection of IgM and IgG antibody

This test is commercially available and can detect both IgG and IgM antibodies against dengue with the same kit. Perform the test following the instructions given by the manufacturer along with the diagnostic kit.

Serological tests done in reference laboratory

Following serological tests are done in reference laboratories:

MAC-ELISA

This measures IgM antibodies in the serum. It is very efficient test method and is valuable for laboratories that perform a high volume of testing.

Haemagglutination-Inhibition test

This is a simple, sensitive and reproducible test which can be easily done and is also not very expensive. It, however, requires pretreatment to remove nonspecific inhibitors and paired sera are required for interpretation.

Neutralization test

The most sensitive and specific method is the serum dilution versus constant plaque reduction test.

Dot-blot immunoassay

This is a commercially available kit for detection of dengue antibody.

Reporting of results

➢ Detection of IgM antibody	Positive
➢ A four fold rise in paired sera for HI antibody collected 10 days apart	Positive
➢ A very high titre (>1280) in single serum sample	Strongly suggestive
➢ No change in serum HI antibody in paired sera collected 7-10 days apart	Negative
➢ No IgM antibody in paired serum samples	Negative
➢ Positive culture/antigen detection	Positive

Quality assurance

- ➢ Observe internal quality control methods covering collection, transportation, equipment, reagents and training of technical staff.
- ➢ Laboratory should participate in a quality assurance programme for dengue serology.

Biosafety

- ➢ Safe laboratory techniques as outlined in Chapter 8 must be practised.
- ➢ Use of personal protective equipment, appropriate methods for collection and transportation, proper procedures for serum separation and decontamination and disposal of potentially infectious materials be strictly adhered to.

Referral

The specimens are to be sent to the reference laboratories for:

- ➢ Confirmation of diagnosis
- ➢ Isolation and characterization of virus
- ➢ In outbreak situations
- ➢ Part of Quality Assurance Programme

Further reading

1. Dengue haemorrhagic fever: Diagnosis, treatment, prevention and control. WHO, Geneva, 2nd Ed, 1997.
2. Viral haemorrhagic fevers. Report of a WHO Expert Committee, WHO Technical Report Series No 721, 1985.
3. Lam SK Dengue haemorrhagic fever 6:39,1995.

Index

Acid fast staining 17

AIDS 157
- Collection of sample of blood 158
- Guidelines for HIV testing 161
- Quality control 159
- Referral 159
- Reporting of results 160

Aminoglycosides 47

Antimicrobial susceptibility testing 43
- Modified Kirby-Bauer method 44
- Quality assurance in susceptibility test 49
- Biosafety 50
- Referral 50
- When intermediate laboratories need not undertake susceptibility tests? 51
- Salient features of quality control in antibiotic susceptibility testing 51

Bacillary dysentery 95

Bacillus cereus 153

Bacteriological media 23
- Types of media 23
- Preparation of media and checking of pH 24
- Nutrient broth 24
- Nutrient agar 25
- Glucose broth 25
- Blood agar 25
- Chocolate agar 25
- XLD agar 25
- Buffered glycerol saline 26
- Loeffler serum medium 26
- Blood tellurite agar 27
- Salt broth (10%) 27
- Peptone water 28
- Alkaline peptone water 28
- MacConkey agar 28
- Bile salt agar 28
- Selenite F broth 30
- Media for carbohydrate fermentation 30
- Lowenstein-Jensen medium 31
- Cary and Blair transport medium 32
- Stuart transport medium 32
- Venkataraman-Ramakrishnan (V.R.) holding medium 33
- MacConkey broth for bacteriological examination of water 33
- Performance of plated media 36

Safety in laboratories 53
- Laboratory biosafety levels 53
- Preventive measures against laboratory infections 54
- Accidents in laboratory 56
- Management of laboratory accidents 57
- General laboratory directions for safety 58

Blood culture 79

Cephalosporins 47

Cholera 67
- Causative organism 67
- Specimens 67
- Collection, storage and transportation of specimens 67
- Materials 68
- Procedure 69
- Quality assurance 72
- Antimicrobial susceptibility testing 72
- Reporting of results 72
- Referral 72
- Biosafety 72

Clostridium
- botulinum 153
- difficile 153

Coagglutination 91

Corynebacterium 73

Corynebacterium diphtheriae 74

Dengue fever and Dengue haemorrhagic fever 173
- Laboratory diagnosis 173
- Recognizing cases of DF/DHF/DSS 175
- Reporting of results 176
- Quality assurance 176
- Biosafety 176
- Referral 176

Diphtheria 73
- Collection and transportation of specimen 73
- Storage 74
- Processing of swabs 74
- Staining of smears 74

Reporting results 76
Quality assurance 76
Other significant organisms isolated from throat swabs 76
Important points about diagnosis of diphtheria 76
Susceptibility to antimicrobial agents 77
Biosafety 77
Referral 77

Disinfection 13

Dysentery 95
Collection of faeces 95
Macroscopic examination 96
Microscopic examination 96
Isolation of organism 96
Cautions 97
Biosafety 97
Referral 97
Antimicrobial sensitivity testing 97
Reporting of results 97
Quality assurance 98

Enteric fever 79
Isolation of organism 79
Antimicrobial susceptibility testing 81
Serodiagnosis 81
Diagnosis of chronic typhoid carriers 84
Biosafety 84
Referral 84

Food poisoning – bacterial 153
Causative agents 153
Collection of specimen 153
Transportation of specimen 154
Interpretation 155
Referral 155
Quality assurance 155
Biosafety 156

Fungi 144
Cryptococcosis 144
Dermatophytes 144

Gonorrhoea 99
Causative organism 99
Specimen collection 99
Specimen transportation for culture 101
Culture 101
Biosafety 103
Quality assurance 103
Reporting of results 103
Antimicrobial susceptibility testing 103
Referral 104

Haemophilus influenzae 87

Hepatitis A virus 163

Hepatitis B virus, 163

Hepatitis C virus 163

Hepatitis D virus 163

Hepatitis E virus 163

HIV 158

Laboratories
Organization and functions 1
Peripheral laboratory services 1
Intermediate laboratory services 3

Malaria 119
Preparation of blood smear 119
Recognition of the malaria parasite 121
Common defects in making blood films 123
Reporting of results 123
Referral 123
Quality assurance 123
Biosafety 124

Maurer's dots 125

McCrady's table 149

Meningitis-pyogenic 87
Causative organisms 87
Collection, storage and transportation of specimens 88
Abnormalities associated with pyogenic meningitis 89
Immunological tests 91
Antimicrobial susceptibility testing 92
Quality assurance 93
Biosafety 93
Referral 93

Mycobacterium tuberculosis 113

Mycological techniques 141
Collection and processing of samples 141
Presumptive identification based on direct microscopy 143
Biosafety 145
Quality assurance 145
Reporting 146
Referral 146

Neisseria gonorrhoeae 99

Neisseria meningitidis 87

Optochin sensitivity test 90

Parasitological examination of faeces 133
Collection of faecal sample 133
Transportation of samples 134
Macroscopic examination 134
Microscopic examination (temporary wet mounts) 134
Concentration techniques 135
Biosafety 138
Disposal of morbid material 138

Quality assurance 138
Reporting of results 138
Referral 138

Poliomyelitis 167
Collection of stool specimen from a case with acute flaccid paralysis (Poliomyelitis) 167
Reporting of results 168
Referral 169
Quality assurance 169
Biosafety 169

Quality Assurance 59
What is the objective of QA? 59
Factors affecting the quality 59
Quality control of equipment 61
Quality control of culture media 62
Quality control for commonly used tests 64
Quality control of immunological tests 64
QC of diagnostic antigens & antisera 64
QA of antibiotic susceptibility testing 63
In service training of staff 65
Participation in external quality assessment 65

Quinolones 48

Salmonella 79

Schuffner's dots 123

Shigella 95

Collection and transportation of clinical specimens 5
Criteria for rejection of specimens 5
Collection of specimens 6
Transportation of specimens 9

Staining techniques 17
Methylene blue staining 17
Gram staining 18
Albert staining 19
India ink staining 20
Ziehl Neelsen staining 20
Iodine staining for ova and cysts 21
Quality control of stains 22

Sterilization 11
Sterilization by heat 11
Biohazard waste management 15

Syphilis 105
Direct demonstration 105
Reporting of results 107
Susceptibility testing 107
Serological evidence 107
Biological false positives in VDRL 109
Quality assurance in VDRL testing 110
Referral 110

Tetracyclines 48

Treponema 106

Treponema pallidum 106

Tuberculosis 113
Morphology of *M.tuberculosis* 113
Microscopy of sputum 113
Collection of sputum sample 113
Storage and transportation of specimens 114
Preparation of smear and Ziehl Neelsen staining (AFB staining) 114
Culture for M. tuberculosis 115
Biosafety 116
Quality assurance 117
Susceptibility to antituberculosis drugs 117
Reporting of results 117
Referral 117

Tyndall effect 89

Typhoid carriers 84

Universal biohazard sign 56

Urinary Tract Infection 127
Causative organisms 127
Specimen collection 127
Specimen transport 129
Culture 129
Quality assurance 130
Susceptibility to antimicrobial agents 130
Reporting of results 130
Referral 131
Biosafety 131

VDRLtest 107

VDRL,biological false positives 109

Vibrio cholerae 67

Vibrio parahaemolyticus 153

Viral hepatitis 163
Collection and transportation of specimens 163
Reporting of results 165
Quality assurance 165
Biosafety 166
Referral 166

Water, bacterial quality 147
Microbiological examination of water 147
Frequency of examination 151
Standards 151
Reporting 152
Referral 152
Quality assurance 152

Widal test 81

Ziehl Neelsen staining 20

Ziemann's dots 122

World Health Organization
Regional Office for South-East Asia
New Delhi